

# Uracil-DNA in *Drosophila melanogaster*

Ph.D. thesis

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## Abbreviations

2-ohA	2-hydroxyadenine
5'FU	5-fluorouracil
5'P	5'phosphate end
8-oxoG	7, 8-dihydro-8-oxoguanine
A	adenine
AID	activation-induced cytosine deaminase
AP site	apurinic/apyrimidinic site
APF	after puparium formation
BER	base excision repair
BRC	Biological Research Center, Szeged
C	cytosine
caU	5-carboxyuracil
CBP/p300	CREB-binding protein
Ct	threshold cycle
CyO	curly of oster
dCMP	2-deoxycytidine-5-monophosphate
DDR	DNA damage response
DNA	deoxyribonucleic acid
dRP	5-deoxyribose-5-phosphate
DSB	double strand break
dsDNA	double-stranded DNA
dT	thymidine
dTMP	2-deoxythymidine-5-monophosphate
dTTP	2-deoxythymidine-5-triphosphate
dUMP	2-deoxyuridine-5-monophosphate
DUT	dUTPase, dUTP pyrophosphatase
DUT-M	human dUTP pyrophosphatase, mitochondrial isoform
DUT-N	human dUTP pyrophosphatase, nuclear isoform
dUTP	2-deoxyuridine-5-triphosphate
dUTPase	dUTP pyrophosphatase
Endo IV	endonuclease IV-type AP endonuclease
Exo III	exonuclease III- type AP endonuclease
eYFP	enhanced yellow fluorescent protein
FADD	Fas-associated death domain protein
FdUTP	fluorodeoxyuridine triphosphate
foU	5-formyluracil
FUdR	5-fluorodeoxyuridine
FUTP	5-fluoro-UTP
G	guanine
GFP	green fluorescent protein
hmU	5-hydroxymethyl uracil
hoU	5-hydroxyuracil
hpRNA	hairpin RNA
HR	homologous recombination
hTDG	human TDG
Hx	hypoxanthine
IC50	half maximal inhibitory concentration

IR	inverted repeat
MBD4	methyl-CpG binding protein 4
<sup>me</sup> C	5-methylcytosine
MED1	methyl-CpG-binding endonuclease 1
MLH1	MutL homolog 1
MMR	mismatch repair
MPG	methylpurine-DNA glycosylase
MUG	mismatch uracil glycosylase
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NLS	nuclear localization signal
OGG1	8-oxoguanine glycosylase
PCNA	proliferation nuclear antigen
PCR	polymerase chain reaction
PKC	protein kinase C
PNK	polynucleotide kinase
Polβ	DNA polymerase β
RFC	replication factor C
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RPA	replication protein A
S2	Drosophila Schneider 2 cells
s.e.m.	standard error of measurement
siRNA	short interfering RNA
SMUG	single-strand selective monofunctional uracil-DNA glycosylase
SSB	single-strand break
ssDNA	single-stranded DNA
T	thymine
TDG	thymine-DNA glycosylase
Tg	thymine glycol
Thd1	TDG homologue of <i>Drosophila melanogaster</i>
Thp1	TDG homologue of <i>Schizosaccharomyces pombe</i>
TS	thymidylate synthase
U	uracil
UAS	upstream activator sequence
UGI	uracil-glycosylase inhibitor
UMP	uridine-5-monophosphate
UNG	uracil-DNA N-glycosylase
VDRC	Vienna Drosophila RNAi Center
XP	xeroderma pigmentosum

## Prologue

The present study aimed to discuss different aspects of uracil emerging in DNA. It will give a detailed overview about its physiological impact, and about uracil-DNA induced cellular response. Furthermore, significance of uracil-DNA associated mechanisms and enzymes are assessed. My research has attempted to evaluate the impact of uracil-DNA in *Drosophila melanogaster*, with special emphasis on its life cycle. In advance, Figure 1 highlights the main subjects of this thesis.

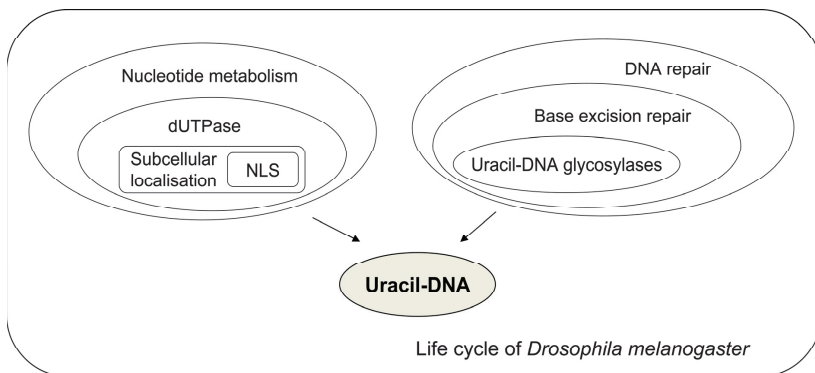


Figure 1 Uracil-DNA and related processes are the main subjects of this thesis

## Introduction

### Uracil in RNA and in DNA

Generally, flow of genetic information follows the DNA to RNA to protein scheme according to the central dogma of molecular biology. DNA encodes, stores, and transmits the genetic instructions required for cellular functions. Information encoded in DNA is translated into RNA and protein levels which then possess structural, regulatory and enzymatic functions that are required for development, differentiation and normal cellular processes.

From the chemist's point of view, DNA is a negatively charged polymer which forms a double helix structure. Building blocks of DNA are deoxyribonucleotides with four different bases: adenine, guanine (pyrimidine bases) and thymine and cytosine (purine bases) (Figure 2). Within the helical structure of DNA, adenine forms base pairs with thymine and guanine with cytosine through hydrogen bonds, thus sequence of one strand of DNA completely determine the sequence of the other strand [1].

Contrary to DNA, RNA usually exists as a single stranded macromolecule where double stranded segments may also form. Regarding its chemical composition, in the sugar-phosphate backbone of RNA ribose is present instead of deoxyribose, and uracils substitute thymine bases (Figure 3). The only difference between thymine and uracil is a methyl group in 5' C atom position. Therefore thymine is actually methylated uracil, and uracil is a thymine analogue [1].

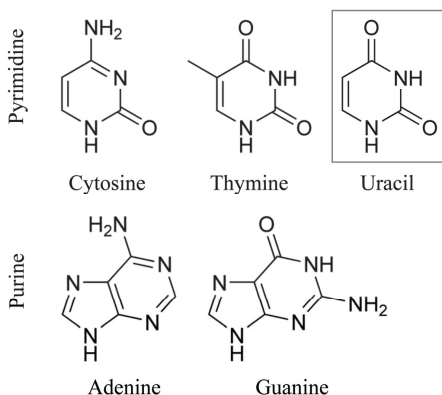


Figure 2 RNA and DNA bases. Uracil is present almost exclusively in RNA.



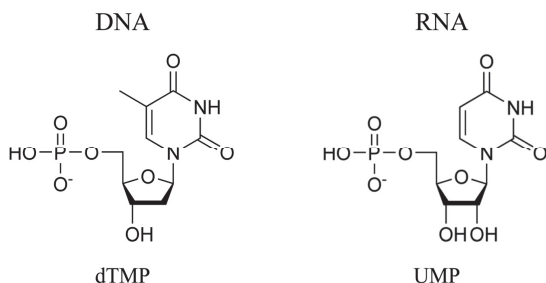


Figure 3 Thymine vs. uracil and deoxyribose vs. ribose exchanges are the major chemical differences between DNA and RNA.

Textbooks say that uracil is not a native component of DNA. However, it occurs with relatively high frequency [2]. These emerges are due to endogenous cellular processes such as dUMP incorporation during replication or spontaneous cytosine deamination (Table 1). In most cases, appearances of uracil are regarded as DNA damage and induce DNA repair mechanisms specific to uracil elimination [3].

Incorporation of uracil during DNA synthesis is the main source of uracil in DNA. As most DNA polymerases are not able to distinguish between dUTP and dTTP, only dUTP/dTTP concentration ratio would determine which nucleotide is more likely to build in to the new DNA strand. If dUTP is used instead of dTTP (thymine-replacing uracil incorporation), uracil in the newly synthesised DNA forms base pair with adenine in the template strand. In this case uracil “contamination” does not change the genetic information, it is not mutagenic.

In contrast to this, deamination of cytosine would lead to G:C to A:T transition mutations (Figure 4 and Figure 5), the most abundant base substitution mutation observed in aerobic organisms [2, 4]. If uracil within the G:U context is not corrected, opposite to uracil an adenine will be incorporated during the subsequent round of DNA replication.. Deamination of cytosine generates uracil with a frequency of 100 – 500 per day in the genome of a human cell [2, 5], thus it has a high mutagenic potential.

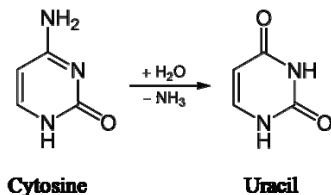


Figure 4 Deamination of cytosine

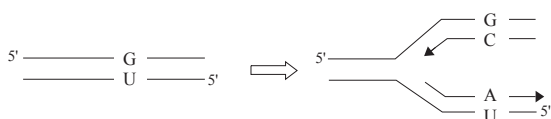


Figure 5 Deamination of cytosine is a mutagenic base modification

Mechanism	Original bp	Resulted bp	Effect
Thymine-replacing incorporation	A:T	A:U	Non-mutagenic
Cytosine deamination	G:C	G:U	Mutagenic

Table 1 Source of uracil in DNA

Spherical consequences of uracil in DNA very much depend on the nature of base pairing partner. Calculated base pairing energies and hydrogen bond distances of uracil in DNA were reported [6]. The strength of the G:U interaction is  $10 \text{ kcal mol}^{-1}$  less than the interaction in the Watson–Crick G:C base-pair, and the distance between G and U is somewhat different from G-C distance. Calculations further support that uracil arisen from dUMP incorporation serves equivalent analogue of thymine if it paired with adenine, because A:U and A:T possess the same interaction energies and hydrogen bond distances [6].

Uracil arisen from either dUMP incorporation or cytosine deamination is eliminated from the DNA by the action of a set of DNA repair enzymes [7]. Mostly the base excision repair (BER) mechanism is responsible for uracil repair, which is not able to distinguish between harmful uracil (cytosine deamination born) and neutral uracil (dUMP misincorporation born). dUTPase prevents the incorporation of dUMP, by efficiently reducing the dUTP pool available for DNA synthesis [3, 8].

## Uracil Repair

### DNA damaging agents, lesions and DNA repair processes

Genomes are vulnerable to an array of DNA-damaging agents, of both endogenous and environmental origin [9]. Damages could cause single base substitutions, chromosome aberrations and block of replication or transcription etc., thus leading to severe defects in cellular function. These lesions of DNA contribute to diseases, carcinogenesis and ageing [2, 10-12]. Several evidences support that accumulations of DNA damages are the main source of cancer development, where mutation in genes involved in cell cycle regulation and DNA repair further leads to progression of malignant condition. According to the nature of damage, specialised DNA repair processes have been evolved to restore the integrity of genetic material [5].

DNA damages arisen from endogenous metabolic processes occur at significant rates *in vivo*. Hydrolysis, oxidation, alkylation and deamination represent the most significant adverse effects to DNA [9] (Figure 6). Spontaneous hydrolysis of the N-glycosylic bond generates an abasic site, also termed as apurinic/apyrimidinic site (AP site). It has been suggested that up to 10,000 bases are lost per day per mammalian cell owing to hydrolytic depurination [13]. Attack of reactive oxygen species (ROS) generated as by-products of normal aerobic metabolism gives rise to many oxidized bases, e.g. 7, 8-dihydro-8-oxoguanine (8-oxoG), 2-hydroxyadenine (2-ohA), thymine glycol (Tg) or 5-hydroxy cytosine [14]. Alkylation of DNA bases constitutes the third large group of endogenous base modification. The intracellular methyl group donor S-adenosylmethionine may covalently modify ring nitrogen residues of DNA bases, resulting in particularly 7-methylguanine and 3-methyladenine [14]. An other base alkylating modification can be generated in DNA as a consequence of lipid peroxidation [15]. In addition, DNA bases are susceptible to hydrolytic deamination, which affects all DNA base residues except thymine, as this latter does not contain an amino group. Deamination converts cytosine to uracil, 5-methylcytosine (<sup>m</sup>C) to thymine, guanine to xanthine and adenine to hypoxanthine. By comparison, deamination of purines is a minor reaction while deamination of cytosines in G:<sup>m</sup>C or G:C base pairs occurs relatively frequently resulting in G:T or G:U mispairs respectively [2]. By comparing deamination rate of <sup>m</sup>C and C, it was shown that 5-methylcytosine can be deaminated three to four times more rapidly than cytosines [8]. In addition, G:T or G:U pairs are repaired through different DNA

repair systems: uracil is very rapidly recognised and excised by uracil-DNA glycosylases, while the G:T basepair in DNA is a substrate for the less effective mismatch-specific repair system (MMR) [2]. Therefore it is suggested that <sup>me</sup>C deamination could be a major contributor to C-T transition at CpG sites, even though <sup>me</sup>C represents only 2-5% portion of all cytosines [2]. The double threat lurking in <sup>me</sup>C deamination is that such modification could not only alter genetic information, but also affect the epigenetic status.

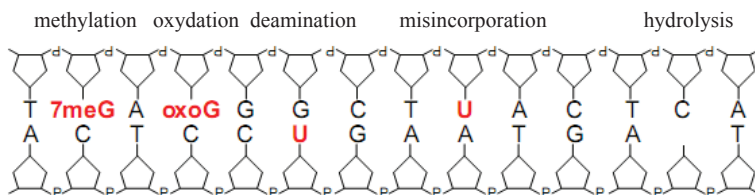


Figure 6 Endogenously generated base modifications [16]

During replication, misincorporation of nucleotides would lead to mismatches at a relatively high rate, but the proofreading activity of DNA polymerase cleaves these mistakes off by utilizing its 3'-5' exonuclease activity [1]. Only dUMP could stay permanently in A:U pairs of newly synthesised DNA, because for most DNA polymerases, uracil is akin to thymine. Generally, base excision repair (BER) is responsible for the elimination of damaged bases or base losses, and MMR corrects mispairs. During replication, the structure of DNA is particularly exposed to various damages. Notably single strand break (SSB) may occur, especially upon ROS attack, leading to collapse of replication fork [2]. These SSBs are also repaired via enzymes acting in later stages of BER. The beauty of BER lies in its ability to handle numerous chemically distinct damages by making their repair converged to similar intermediates.

Large number of various environmental mutagens exists; such as UV radiation, X-ray, radioactivity and a wide variety of chemical compounds. Still, under normal circumstances, DNA damage arisen from endogenous origin is a more frequent event, than exogenous damage [17]. UV light is the most important environmental mutagen [18]. It causes the

formation of dipyrimidine photoproducts, principally cyclobutane pyrimidine dimers and (6-4) photoproducts, which are covalent modification of two adjacent pyrimidin residues [10]. These lesions are highly toxic and mutagenic to cells. Mutations that inactivate tumor suppressor genes, for example p53 often exhibit the signature pattern of UV-induced sequence changes in the DNA of skin cancer cells [9]. Presence of photoproducts yields torsion in DNA structure which serves as a basis of damage recognition for the nucleotide excision repair pathway (NER) [19]. Defects in NER pathway in human cells lead to the severe inherited disease xeroderma pigmentosum (XP), which is a well-characterised example of DNA repair deficient condition [20].

Many exogenous chemical compounds react with DNA causing bulky adduct formation with characteristic DNA structure distortion, constituting a substrate for NER [9]. Alkylating agents, aromatic substances, heavy metals could be harmful for DNA and cells. Planar aromatic compounds can react with DNA by intercalation, therefore block transcription.

The most severe DNA damage is double strand break (DSB), as a consequence of which a fragment of chromosome, i.e. large amount of genetic information could be lost. Ionizing radiation emerging upon, for example radioactive decay, and X-ray radiation are deleterious for cells because they may induce DSB. To counter this treat, free DNA ends are effectively rejoined by homologous recombination (HR) or non-homologous end joining (NHEJ) repair processes [21]. During inherent homologous recombination or upon the action of abortive topoisomerase I and topoisomerase II action, DSBs are also introduced endogenously [5].

DNA damaging agents could introduce mutations to DNA, and in this way initiate cancer development. However, they are also widely used to treat cancer in chemotherapy and radiotherapy clinical strategies. In therapeutic applications DNA damaging is supposed to reach such a toxic degree that provokes cell death even in cancer cells.

In addition, DNA damages can induce diverse reactions in cells, with far-reaching consequences, termed as DNA damage response (DDR) signalling, which is far more complex than sole DNA repair. DNA damage signalling has large impact on cell cycle regulation, cell fate decision (apoptosis/senescence), transcription regulation and DNA repair [5, 22].

Damaging agent			
Oxygen radicals	UV light	IR	Replication error
Alkylating agent	Polycyclic aromatic hydrocarbons	Chemicals	Deamination
Deamination		X-ray	
Hydrolysis			
Misincorporation			
Lesion			
Abasic site	Dipyrimidine photoproducts	Double strand break	A:G mismatch
Uracil	Bulky adducts	Interstrand crosslinks	T:C mismatch
8-oxoguanine			Insertion
Single strand break			Deletion
Repair process			
Base Excision Repair	Nucleotide Excision Repair	Homologous recombination	Mismatch Repair
Transcription-coupled repair		Non-homologous end joining	

Table 2 Damaging agents, DNA damage and DNA repair

### Base excision repair

Base excision repair (BER) is perhaps the most fundamental and ubiquitous DNA repair mechanism in all higher organisms. It counteracts the mutagen effect of numerous minor alterations of bases mostly emerging from endogenous origin [14]. Consequently, BER possesses a major role in maintaining genetic integrity of the genome. For example, the human base excision DNA repair pathway repairs ~10,000 lesions per cell per day [2].

BER is a multiple-step mechanism which is initiated by detection and excision of an altered base residue in free form by a DNA glycosylase. DNA glycosylases cleave the base-deoxyribose N-glycosylic bond of a damaged nucleotide residue leaving an abasic site behind (Figure 7) [23]. The abasic site, which may also occur due to endogenous hydrolysis, serves as a common intermedier in BER processes of chemically distinct DNA base damages [9]. In a subsequent step AP endonuclease or AP lyase make an incision at the AP site (Figure 8) [14]. AP endonuclease catalyses the 5'-phosphodiester hydrolysis leaving a nick with a 3'-OH and a 5'-deoxyribose 5-phosphate (dRP) end. Contrary to this, AP lyase cleaves the DNA backbone on the 3' side of the AP site. Both reactions generate a single strand break [14]. Next, repair process is completed with repair synthesis and ligation. These later steps can proceed by short patch or long patch pathway of BER involving different protein partners

(Figure 9). The distinction is based on the number of newly incorporated nucleotides: in the short patch only one nucleotide is sufficient to fill the gap, while during long-patch BER 2-8nt stretches are synthesised beginning at the damaged site [24]. In most cases, BER follows the short-patch pathway, when DNA Pol $\beta$  (polymerase  $\beta$ ) induces elimination of the 5'-phosphate at the AP site via its dRP-lyase activity and the final ligation step is carried out by DNA ligase III in partnership with the scaffold protein XRCC1 [24]. If the long-patch pathway is employed, dRP moiety in conjunction with the sugar residue is removed by flap endonuclease I (FEN1) [14]. Thus strand displacement synthesis does no longer require dRP-lyase activity of DNA polymerase  $\beta$ . In long patch BER, repair synthesis can be carried out by Pol  $\delta$  and Pol  $\epsilon$  in addition to Pol  $\beta$ . For this process, it is essential that Pol $\delta$  and Pol $\epsilon$  are in interaction with proliferation nuclear antigen (PCNA) and replication factor C (RFC). PCNA has an important role in long-patch BER; it recruits FEN1 and DNA ligase I to the AP site and stimulates nuclease activity of FEN1 [14].

Participants of both short- and long-patched BER are conserved from *E.coli* to mammals; however absence or presence of particular enzymes may differ from species to species.

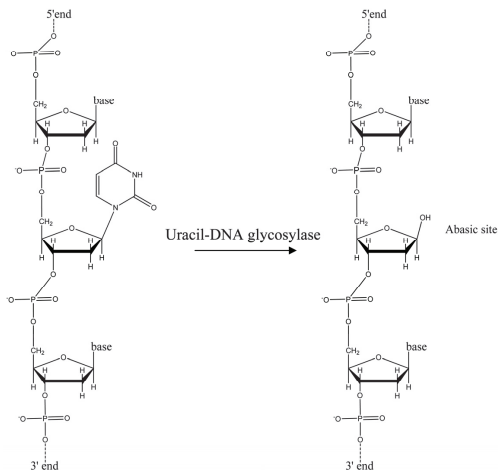


Figure 7 DNA glycosylases cleave the base–deoxyribose N-glycosylic bond of a damaged nucleotide residue leaving an abasic site behind. Figure 7 shows uracil removal from DNA by uracil-DNA glycosylase.

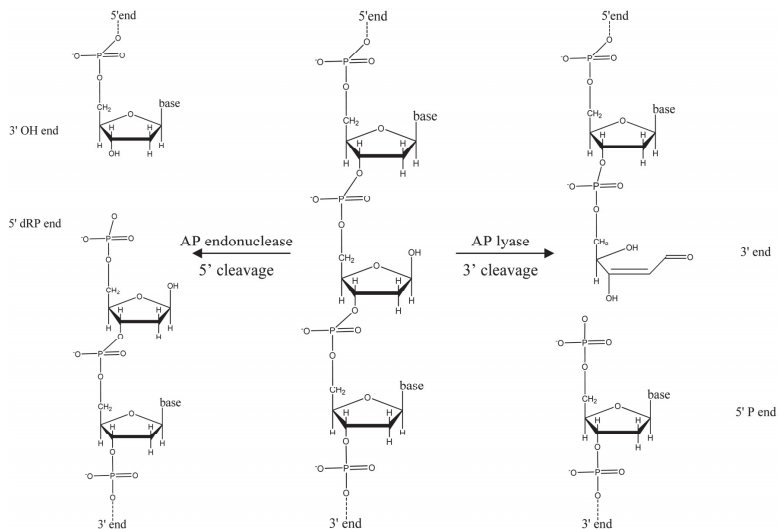


Figure 8 AP endonuclease and AP lyase can make an incision at the AP site generating 5'-deoxyribose 5-phosphate (dRP) end or 5'-phosphate (5'P) end respectively

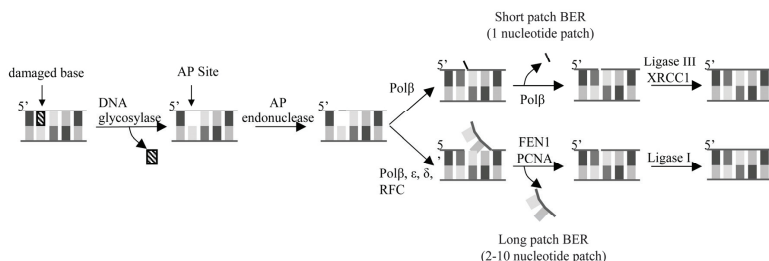


Figure 9 An outline of the BER pathway, including the two subpathways known as the short-patch and long-patch repair pathways. (Reproduced with modifications from Schultz-Norton *et al.* [25])



Several DNA glycosylases have been identified and their substrate specificities have been described [14]. Each acts on a limited number of modified or damaged bases. BER is unique among the DNA repair processes in that the individual base lesions are recognized by distinct enzymes. Four enzymes have been identified in human cells which are responsible for oxidised base lesion repair [14]. The 8-oxoguanine glycosylase (OGG1) removes oxidized purines (8-oxoG and ring-opened guanine, i.e., formamidopyrimidine) and NTH1 removes oxidised pyrimidines (T/C-glycol, dihydrouracil). Mammalian specific DNA glycosylases, NEIL1 and NEIL2 target for example, ring-opened purines, T-glycol and 5-hydroxyuracil, and preferentially excise base lesions from single-stranded DNA [14]. NEILs induce a PNK-dependent unique BER subpathway, which does not need AP endonuclease as NEILs have  $\beta\delta$  lyase activity and polynucleotide kinase (PNK) is also able to remove 3'phosphate in addition to its 5'kinase activity [14]. If oxidised guanine (8-oxoguanine) forms mismatch base pair with adenine, it is processed by MYH, the *E.coli* homologue of MutY [8]. Interestingly it removes adenine and not 8-oxoguanine. Methylpurine-DNA glycosylase (MPG) shows a broader spectrum of substrate preference, it is able to cleave off a wide range of damaged purines, even undamaged purines at low rate [26]. Lastly, uracil, the focus and object of this dissertation, is removed by members of the uracil-DNA glycosylase enzyme family that could be categorised into at least 4 subfamilies: UNG, SMUG, TDG and MBD4 [14]. Some of the DNA glycosylases also have a mitochondrial isoform, which aim to counteract higher rate of oxidative damage present in the site of endogenous ROS production [27].

DNA glycosylases need to search for DNA damages within the cell. Considering the frequency of damage occurrence in a human-sized genome, DNA glycosylases actually look for a needle in a haystack, when scanning DNA sequences. Sliding along one strand would not be sufficient on its own to explain the entire mode of damage locating. It is suggested that DNA glycosylases hop (microscopic dissociation and re-association) and slide (rotational diffusion) in order to monitor both strands accurately and use hopping for strand switching [28]. In *E. coli*, uracil-DNA glycosylase is dominated by hopping over long distances with local sliding contributing to damage recognition [28].

Despite the differences in the chemical nature of DNA damages corrected by different DNA glycosylases, they apply similar strategy for their recognition. They induce an extrahelical flipping of the damaged deoxynucleotide into a lesion-specific recognition pocket. All DNA glycosylases studied so far bind to the minor groove, bend DNA at the site of damage, and flip the lesion base out of the DNA major groove [2, 29]. The initial recognition apparently exploits the deformability of the DNA at a base pair destabilized by the presence of a lesion.

If the damaged base fits into the binding pocket, it can remain inside and can provide the necessary contacts and orientation for its excision.

DNA glycosylases are relatively small (~30–50 kDa) monomeric proteins that do not require cofactors for their activity [30]. According to their enzymatic properties, two groups can be distinguished. The monofunctional glycosylases excise the substrate base, leaving an intact AP site [14]. In contrast, the bifunctional glycosylases have an additional lyase activity to cleave the DNA backbone on the 3' side of the AP site. Lyase activity is characteristic to DNA glycosylases specific for oxidized bases [14]. Mechanistic differences between the two group appear in the source of the nucleophile that attack sugar C1' of the targeted nucleotide [31]. Monofunctional DNA glycosylases, such as uracil-DNA glycosylases and MPG, typically use an activated water molecule as a nucleophile [32]. In the case of bifunctional DNA glycosylases, OGG1, MYH, NTH and NEILs, the nucleophile is often an activated  $\epsilon$ -NH<sub>2</sub> of a lysine or the N-terminal proline in the active site, which are also involved in the subsequent lyase reaction step [14, 24].

The release of altered bases is followed by abasic site repair initiated by AP endonuclease. There are two distinct families of hydrolytic AP endonucleases: exonuclease III- type (Exo III) and endonuclease IV-type (Endo IV) (Table 3) [33, 34]. Activity of Exo III members is dependent on Mg<sup>2+</sup> but Endo IV acts in Mg<sup>2+</sup>-independent fashion [35, 36]. These latter are Zn-enzymes and employ tightly bound Zn ions within their active sites [37]. In *E.coli*, Exo III comprises the major AP endonuclease activity [38], while in the yeast *S.cerevisiae* Apn1, the endo IV homolog, does so [39]. In mammals, only members of the Exo III family have been identified (APE1 and APE2) [40, 41].

	Endo IV family	Exo III family
<b>E.coli</b>	Endo IV	Exo III
<b>S.cerevisiae</b>	Apn1	Apn2
<b>C.elegans</b>	APN-1	EXO-3
<b>D.melanogaster</b>	-	Rrp1
<b>H.sapiens</b>	-	APE1, APE2

Table 3. AP endonucleases

As outlined previously, later steps of BER are gap tailoring, repair synthesis and ligation. Participants and mechanisms of these steps are not discussed in more detail in this summary of BER.

Mutations arisen due to base losses and modifications are implicated in cancer and ageing [11]. Although BER plays a major role in repair of such damages, the functional significance of BER in prevention of disease remains unclear. To date, only deficiencies of nucleotide excision repair (NER) and DNA mismatch repair (MMR) have been associated with diseases (xeroderma pigmentosum and Cockayne syndrome) and cancer. Experimental evidences indicate that lack of single DNA endonucleases would not give strong phenotype in mammals [42-44]. Mouse mutants and their embryonic fibroblasts lacking OGG1, NTH1 and NEIL1 as well as MYH are viable [45, 46]. Furthermore, nor dramatically increased cancer development, neither accelerated aging were observed [46]. One possible explanation for the lack of phenotype of the glycosylase mutants is that, unlike in NER or MMR, multiple glycosylases could substitute others in function because of their overlapping substrate range. Such back-up mechanism could be suggested for uracil-DNA glycosylases as well, of which 4 representatives are present in mammals and 3 in *Drosophila melanogaster*.

## Uracil-DNA glycosylases

Uracil-DNA glycosylases catalyse the reaction of N- glycosylic bond cleavage of uracil in order to clear DNA from its presence [32]. Removal of uracil by members of uracil-DNA glycosylases is important in life, enzymes with UDG activity were described in all domains: Archaea, Bacteria and Eukarya and also some viruses encode UDG [47]. Four subfamilies of UDGs have been characterised in more depth in Eukaryotes. These are UNG (uracil-DNA N-glycosylase), SMUG (single-stranded selective monofunctional uracil-DNA glycosylase), TDG (thymine-DNA glycosylase) and MBD4 (Methyl-CpG binding protein 4) [32]. Table 4 summarizes the presence of uracil-DNA glycosylase subfamilies in several model organisms. In mammals all the four UDG subfamilies can be found, while in *Drosophila melanogaster* UNG is missing. None of the UDG subfamilies is represented ubiquitously [48].

	UNG	SMUG	TDG	MBD4
<b>E.coli</b>	+	-	+	-
<b>S.cerevisiae</b>	+	-	-	-
<b>C.elegans</b>	+	-	-	-
<b>D.melanogaster</b>	-	+	+	+
<b>H.sapiens</b>	+	+	+	+

Table 4 Presence/absence of UDG subfamilies in different model organisms

Uracil DNA glycosylases are monofunctional enzymes, generating an abasic site in the initiation of the BER pathway [24]. Their common characteristic is that they possess the same  $\alpha/\beta$  fold and similar overall structure, although having low sequence homology (8%), indicating that they have evolved from a common ancestor [49]. As differences in structure and sequences occur, distinct substrate specificity are associated with different uracil-DNA glycosylase subfamilies. In addition to uracil, they could repair other uracil-related lesions too, but the context of the lesion is a determinant factor. Although the physiological roles of these glycosylases are not fully defined, given their substrate preferences and their temporally and spatially separated occurrence, it is likely that each of these enzymes have evolved to fulfil specialised functions in genome maintenance. There has been much debate on their potential redundancy and overlapping functions [24].

UNG is the most active among the uracil-DNA glycosylases (and also possibly the most widespread) [50]. It cleaves uracil residues when found in single strand, or double strand DNA paired with adenine or mispaired with guanine; however, only a small number of other pyrimidines are also targets [51]. It recognises uracil explicitly in an extrahelical conformation via flipping-out bases for sampling [29]. Selectivity of UNG towards uracil is achieved via tight shape of the substrate binding pocket and via a set of hydrogen bonding interactions [52]. In the active site two conserved residues, a catalytic asparagine and a histidine participate in the enzymatic reaction [52]. UNG is sensitive to inhibition by a specific bacteriophage PBS protein, UGI, which discriminates its activity from the activity of other uracil-DNA glycosylases [53]. The measured binding affinity of human UNG for uracil-DNA showed an ssU>dsU:G>>dsU:A order *in vitro* [54]. It reflects that, on one hand, uracil in ssDNA is the most easily disrupted, and on the other hand, in duplex DNA, the less stable G:U mismatch is more readily disrupted, than the Watson-Crick A:U base pair. However, it does not indicate that UNG would not favour the repair of A:U base pair. Moreover, current evidence suggests that UNG is the major enzyme responsible for the repair of uracil arising in DNA through dUTP misincorporation [32]. This idea is further supported by the fact that UNG is associated with the replication foci, site of DNA synthesis, where UNG2 co-localizes with PCNA and replication protein A (RPA) [55]. The specific role of UNG2 in the removal of misincorporated uracil has been demonstrated by utilising different experimental setups: first, inhibition of immediate post-replicative removal of incorporated uracil in isolated nuclei was achieved by neutralizing anti-UNG antibodies [55], second, removal of incorporated uracil in nuclei from *UNG*<sup>-/-</sup> mice was found to be slower than in wild type nuclei [56]. However, UNG also removes uracil from U:G mismatches produced by cytosine deamination in DNA. Lack of UNG in *E.coli* and in *S.cerevisiae* gives a mutator phenotype due to increased number of G:C to A:T transitions [57, 58]. Despite the fact that transgenic UGI-expressing human cells (UNG activity is inhibited) also exhibit a high frequency of spontaneous mutations [59], the *ung*<sup>-/-</sup> mice do not show either elevated mutation rate, or increased incidents of cancer development [56]. This finding argues for the existence of other backup enzymes in mammals. Redundancy, however, cannot explain the lack of phenotype in the *ung-1* mutant of *C. elegans*, which is also viable and fertile, showing no adverse effect of lack of ung, since no other uracil-DNA glycosylase is encoded in *C.elegans* [60].

UNG is the only uracil-DNA glycosylase that also has a mitochondrial isoform. Two isoforms, UNG1 and UNG2, are expressed by alternative promoter usage and alternative splicing [61]. They possess different N terminal sequences responsible for subcellular localisation. UNG1 is targeted to the mitochondria, whereas UNG2 is found primarily in the nucleus. In the mitochondria, UNG1 is continuously present, as its expression is driven by a continuous promoter, while expression of UNG2 (nuclear isoform) varies with stages of cell cycle, UNG2 expression is turned on in S phase, when DNA replication occurs [62, 63]. It is also likely that the mitochondrial UNG1 has an important role in the repair of oxidized pyrimidines, and in oxidative stress response.

Recent studies have revealed that UNG has an important role in antibody diversification. It is now established that UNG removes uracil residues in DNA generated by activation-induced cytosine deaminase (AID) as part of somatic hypermutation and class switch recombination processes in activated B-cells [64, 65].

**SMUGs** (single-strand selective monofunctional uracil-DNA glycosylase) are only found in vertebrates and surprisingly in insects [66]. Repair roles of human SMUG1 were assessed by determining its damage specificity and cellular activity. Dual role was associated to SMUGs: on the one hand they repair uracil-DNA serving as a backup enzyme for UDG; on the other hand they are primary repair enzymes for a subset of oxidized thymines that are poorly recognised by other DNA glycosylases [67]. Therefore, in addition to deamination damage, SMUG is also involved in the repair of DNA oxidation damage [67]. Its name is misleading, because it is even more active on mispaired uracil in duplex DNA than in single stranded DNA [68]. In comparison with UNG, SMUG excises uracil from a single-stranded DNA context much less efficiently and it shows strong preference towards U:G mismatch instead of U:A basepair. According to *in vitro* activity measurements, SMUG removes uracil mispaired with guanine appr. 200 times faster than uracil paired with adenine within the same sequence context, indicating that hSMUG1 removes uracil arisen from cytosine deamination rather than from dUTP misincorporation [68]. SMUG: also excises a subject of oxidative base lesions either in ssDNA or in dsDNA [68]. These lesions are damage products of thymine methyl group oxidation, like 5-hydroxymethyl uracil (hmU), 5-formyl uracil (foU), 5-hydroxy uracil (hoU) and 5-carboxy uracil (caU) [67, 68]. All contain the oxidised group attached to the ring C5 position that is very important for damage selection by SMUG. The presence of hydrophilic group in C5 is one of the properties used for discriminating damaged pyrimidines from intact thymine. As intact thymine bears a hydrophobic methyl group at C5 position,

SMUG cannot accommodate it in its active site pocket [66]. The 3D structure of *Xenopus laevis* SMUG was determined for the protein complexed to free aberrant bases [52, 66], which provides exciting insights into catalytic and recognition mechanism of SMUG. It recognises aberrant bases in DNA via specific water bridged (U) or direct (hoU, hmU, foU) hydrogen bonds to the backbone amide NHs [69]. Similarly to UNG, an asparagine and a histidine residue (Asn85 and His239) are crucial for damage excising activity [66]. In respect of the SMUG active site, it also shares common characteristics with TDG concerning residues that lie near the C5 substituent of bases [66]. These residues participate in the coordination of damaged base via H bond formation. It binds tightly to abasic site-containing DNA following the removal of a target base, which causes inhibition of the subsequent enzymatic cycle and probably causes lower catalytic activity than UNG [68]. Contrary to UNG, SMUG can not be inhibited by UGI [70].

*In vivo*, SMUG is expressed during the whole cell cycle at similar levels, and its expression is not notably increased in proliferative tissues [70]. It acts to repair 5-hydroxymethyl uracil arisen from thymine oxidation and uracil arisen from cytosine deamination. It is therefore a backup enzyme for UNG activity indicated also by genetic evidences [70]. UNG-deficient mice embryo fibroblast cells show strong mutator phenotype only upon simultaneous knockdown of SMUG1 expression. Then increased frequency of transition mutations at C:G was observed [70].

Members of **TDG** and **MBD** subfamilies are much less efficient uracil DNA glycosylases than UNG or SMUG. Although there is no apparent amino-acid sequence similarity between the two groups, they share many common features. Both appear to act exclusively on duplex substrates, with a strong preference for mispaired pyrimidines, such as G:U and G:T, and a strong preference for damage located in CpG dinucleotides. (G:U may arise from cytosine deamination, and G:T from <sup>m</sup>C deamination.) Therefore their possible primary role is to maintain genetic integrity of CpG islands, hotspots for epigenetic regulation through cytosine methylation.

**TDG** is related to a bacterial G:U-processing enzyme, mismatch uracil glycosylase (MUG), although their substrate spectra are not equal [71]. The human TDG excises thymine from G:T and uracil from G:U mismatched substrates, whereas the *E.coli* Mug protein processes G:U substrate but fails to act on G:T mismatches with an appreciable efficiency [71]. Among eukaryotic TDG orthologs the common top substrate is the G:U mispair, however species-specific substrate preference may vary according to the specific needs of the individual

species [71]. Neither the G:T processing activity nor the mismatch dependency of the proteins appear to be strictly conserved. By comparing the substrate specificity of TDG orthologs, hTDG (human TDG), Thd1 (TDG homolog of *Drosophila melanogaster*) and Thp1 (TDG homolog of *Schizosaccharomyces pombe*), it has been revealed that only the human enzyme processed G:T mismatch at a physiologically relevant rate [71]. For the *Drosophila* Thd1 G:T mismatch was a poor substrate and *S.pombe* Thp1 even refused to act on this substrate [71]. Contrary to the mammalian and *Drosophila* enzymes, Thp1 showed little preference for mismatched substrate and processed U:A or uracil in an ssDNA context with remarkable efficiency [72]. The differences in substrate specificity are consistent with sequence divergence. Above the previously discussed substrates, TDGs could also accommodate hypoxanthine (Hx) in their relatively loose substrate binding pocket, and mediates repair of G:Hx pairs [71].

Instead of asparagine and histidine residues, two asparagines are essential for the catalysis of the N-glycosylic bond cleavage. The TDG subfamily of UDGs is not inhibited by UGI [73]. Similarly to SMUGs, tight binding to the product AP-site causes practically complete product inhibition [71].

TDG is not present throughout the entire cell cycle. It is absent from S-phase due to degradation by the proteasome system at the G1-S boundary [63]. Posttranslational modifications further modulate DNA repair function of TDG. Interplay between acetylation and phosphorylation was recently investigated [74]. Both modification could occur on adjacent residues in the amino-terminus and are mutually exclusive [74]. Remarkably, acetylation by CBP/p300 selectively abolishes G:T processing while phosphorylation by PKC $\alpha$  may preserve this function *in vivo* by preventing CBP-mediated acetylation [74]. Sumoylation, another posttranslational enzymatic modification of TDG has recently been described and characterized [74]. The SUMO conjugation at a unique K330 consensus site located within the C-terminal region of TDG. It is involved in a structural modification of the nearby active site and causes selective prevention of G:T mismatch repair. Consequently, acetylation and sumoylation direct similar effects [75].

Human TDG has furthermore been shown to have a functional role in transcription and epigenetic regulation. It interacts with the retinoic acid and estrogen receptor transcription factors and with SRC-1 regulatory molecule [76].

**MBD4** (methyl-CpG binding protein 4), also known as MED1 (methyl-CpG-binding endonuclease 1), is a mismatch-specific G:T and G:U DNA glycosylase [77]. As its name



implies, MBD4 contains a methyl-CpG binding domain in N-terminal position and further more a C-terminal DNA glycosylase domain [78]. The methyl-binding domain most likely enhances the localisation of the DNA *N*-glycosylase activity to regions of the genome where 5-methylcytosine levels are high and therefore, where <sup>me</sup>C-deamination occurs with highest frequency. Substrate spectrum of human MBD4 protein was described in more details, and revealed that it extends beyond G:T and G:U mispairs [78]. MBD4 also recognises and removes the uracil analogue 5-fluorouracil paired with guanine and thymine glycol (Tg) if paired with guanine, although efficiencies of these lesion removals are about half of that for removal of T:G [78]. Thymine glycol is a major replication-interfering lesion generated by reactive oxygen species, but it is also produced by deamination of 5-methyl-cytosine and then present as a Tg:G mispair [77]. A novel alternatively spliced form of the MBD4 DNA glycosylase was identified recently in HeLa cells, which lacks the methyl-binding domain but retains the glycosylase domain. Surprisingly, this shortened version of MBD4 possesses uracil DNA glycosylase but not thymine DNA glycosylase activity [79].

It is interesting that the function of MBD4 is quite similar to that of TDG, despite the complete lack of sequence homology [77]. Both contribute to mutation avoidance at methylated CpG dinucleotides. Mutagenic mechanisms involving 5-methylcytosine appear to be particularly common since methylated CpG dinucleotides are mutational hotspots in human genes, for example in the cancer-relevant p53 gene [77]. Experimental evidences argue for the protective role of MBD4 at these sites. In *Mbd4* knockout mice, frequency of C to T transitions at CpG sites was increased by a factor of three; and on a cancer-susceptible background, lack of MBD4 has led to accelerated tumor formation indicating that MBD4 suppresses CpG mutability and tumorigenesis *in vivo* [80].

In mammals, MBD4 function extends beyond sole DNA repair as it is involved in DNA damage response too. MBD4 deficiency could reduce the apoptotic response to DNA-damaging agents [81]. It was shown that it interacts with the mismatch repair (MMR) specific MLH1 protein via its glycosylase domain [82] and MMR-dependent apoptosis is mediated through MBD4. In addition, normal apoptotic response to  $\gamma$ -irradiation, which is independent of Mlh1, is also reduced in MBD4 deficiency suggesting that the reliance upon MBD4 may extend beyond MMR-mediated apoptosis [81]. MBD4 has the ability to bind the Fas-associated death domain protein (FADD) that could serve as further link to DNA damage induced apoptotic signalling and response [83].

**In summary**, current view suggests that in mammals UNG and SMUG1 are the major enzymes responsible for the repair of the U:G mispairs caused by spontaneous cytidine deamination, whereas uracil arising in DNA through dUTP misincorporation is mainly dealt with UNG alone. In contrast, MBD4 (and possibly TDG) appear to have a more specialised function in correcting T:G mismatches that arise from deamination of 5-<sup>me</sup>C (especially in a CpG context) [80] (Figure 10, Table 5).

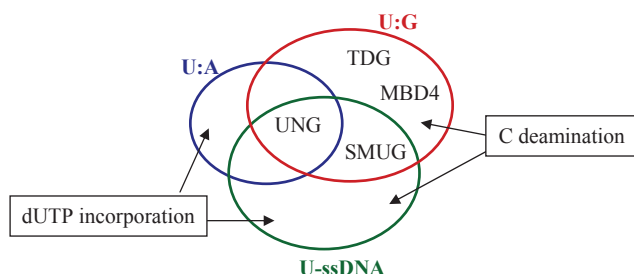


Figure 10 Uracil is processed by different uracil-DNA glycosylases depending on its context in the DNA.

UDG subfamily	substrate specificity	expression pattern	interacting partners
<b>UNG</b>	ssU, A:U, G:U	S phase specific	PCNA, RPA
<b>SMUG</b>	G:U, ssU, oxidised T	whole cell cycle	
<b>TDG</b>	G:U, G:T, G:Hx	not present in S phase	CBP/p300, PKC
<b>MBD4</b>	G:U, G:T, G:Tg	whole cell cycle	MLH1, FADD

Table 5 Characteristics of different uracil-DNA glycosylases

## The significance of nucleotide metabolism and dUTP incorporation

Fine modulation of nucleotide pools is an important aspect of maintaining error-free DNA. Frequency of dUMP incorporation into DNA, thus appearance of U:A pairs, depends largely on the relative sizes of the intracellular dUTP and dTTP pools. dUTPase and thymidylate synthase (TS) are the two major factors responsible for the regulation of dUTP and dTTP levels. dUTPase (dUTP pyrophosphatase) catalyses the hydrolysis of dUTP into dUMP and pyrophosphate in the presence of magnesium ion. (Figure 11)

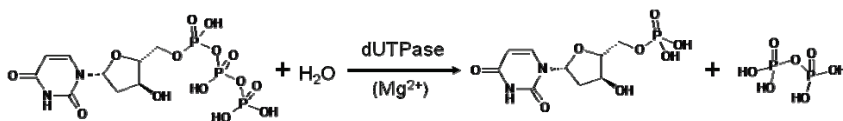


Figure 11 The catalytic reaction of dUTPase: Formation of dUMP and pyrophosphate by the hydrolysis of dUTP.

This reaction has a dual role within the cell. On one hand, it decreases the cellular dUTP level; on the other hand, it provides the precursor of dTTP biosynthesis by producing dUMP. Thymidylate synthase subsequently attach a methyl group to dUMP to produce dTMP, which is phosphorylated up to dTTP in two steps (Figure 12). Thus, dUTPase takes part in the regulation of the metabolism of two nucleotides (dUTP and dTTP) in one reaction, and consequently keeps dUTP/dTTP ratio at low level [84]. In eukaryotes, there exist three possible routes of dTMP, thus dTTP biosynthesis. These all contribute to the regulation of dTTP level (Figure 12). First, as above described, dUTPase with thymidylate synthase can produce dTMP, second, dTMP is produced by consecutive reaction of dCMP deaminase and thymidylate synthase. Last, thymidine kinase could generate dTMP from thymidine (dT). dUTPase, dCMP deaminase and thymidylate synthase take part in the *de novo* pathway of thymidylate metabolism, while thymidine kinase is a key enzyme of the salvage pathway. Due to the complex regulation of nucleotide levels, the cellular dUTP/dTTP ratio varies around  $10^{-2}$ - $10^{-3}$  [85], depending on cell type and cell cycle stage. However, despite this precise regulation, incorporation of uracil into DNA due to the use of dUMP instead of dTMP by DNA polymerases is still the most common type of endogenous DNA damage.

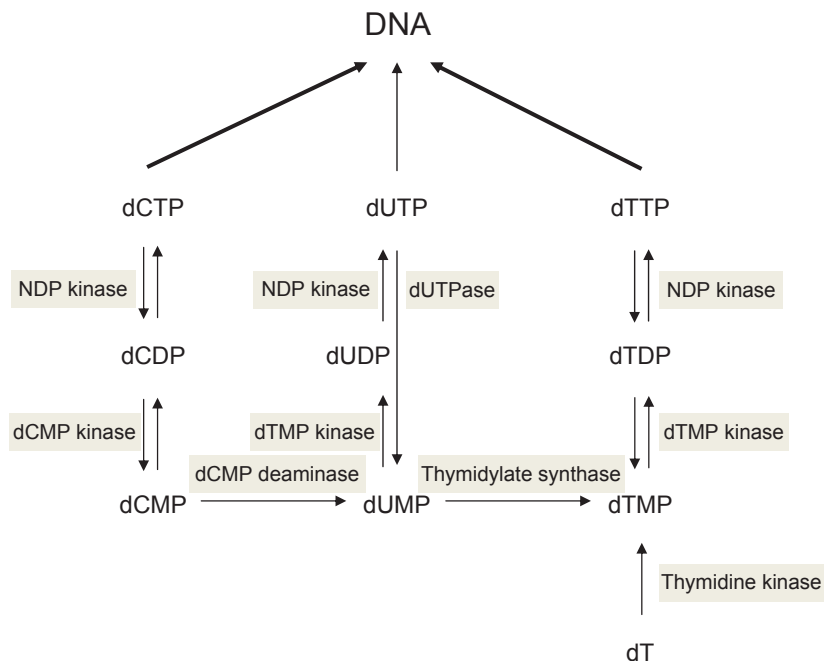


Figure 12 Biosynthetic pathways of pyrimidine deoxyribonucleotides

Upon misregulation, dUTP/dTTP ratio is elevated; therefore dUTP can be incorporated into DNA with high frequency [86]. Repetitive futile attempts of the DNA repair mechanism to eliminate high number of uracil would result in multiple DNA single strand breaks, chromosome fragmentation and cell death. This process was termed as thymineless cell death [87, 88]. Some widely used chemotherapeutic agents (*e.g.*, thymidylate synthase inhibitors such as fluoro-uracil and fluoro-uracil derivatives) were suggested to utilise a similar mechanism by targeting thymidylate metabolism and causing extreme depletion of dTTP pool [89].

Significance of dUTPase in thymidylate metabolism is further demonstrated by the fact that expression level of dUTPase is in correlation with response to thymidylate synthase inhibitor chemotherapy. High nuclear dUTPase expression is associated with poor response, shorter time to progression, and poorer survival. Conversely, low nuclear dUTPase level is associated with response to chemotherapy, longer time to progression, and greater overall survival [90].

## The significance of dUTPase

dUTPase is essential for all organisms, especially in replicating cells. Lack of dUTPase leads to thymineless cell death, as a consequence of UNG induced DNA repair. Lethality upon disruption of dUTPase function was reported in *E.coli*, *S.cerevisiae* and *C.elegans* [91-93]. Viability and adverse effects of absence of dUTPase could be restored when the *ung* gene is disrupted simultaneously. It therefore indicates that uracil removal was the major cause of lethality. In *S.cerevisiae* a viable, but mutant allele of DUT (dUTPase) gene was also identified. This mutant strain showed growth delay and cell cycle abnormalities and exhibited a mutator phenotype [94]. Similar consequences of dUTPase depletion were observed in *C.elegans*, however lack of *clk-2* checkpoint gene could also rescue lethality and developmental defects [93]. Furthermore, *clk-2*  $-/-$  could eliminate cell-cycle arrest and apoptotic response given for dUTPase silencing. These data indicate that CLK-2 dependent DNA damage response pathway is activated after uracil incorporation into DNA. Due to persistent uracil removal, intermediates and/or single-stranded DNA are formed, which induce apoptosis via DNA damage response pathway. This observation extends our previous hypothesis about thymine less cell death, that DNA fragmentation would induce cell death. Moreover, in the absence of the CLK-2 checkpoint repair intermediates are tolerated in *C.elegans* [93].

The impact of dUTPase and precise regulation of dUTP/dTTP pool are also regarded as preventive DNA repair mechanism. Elevated level of uracil in DNA was detected in *dut- ung- E.coli* [95]. The CJ236 strain *E.coli* lacks both *dut* (dUTPase) and *ung* gene, thus it is viable, but contains 3000-8000 uracil/ $10^6$  nucleotide and shows increased mutation frequency due to absence of UNG. This number is extremely high, if compared to uracil content of wild type or UDG mutant *E.coli* strains. In wild type *E.coli*, uracil occurs at a rate below  $1/10^6$  nucleotide, while the CY11 *mug-*, *ung-* strain accumulates 23-33 uracil/ $10^6$  nucleotides. If the CY11 strain was treated with thymidylate synthase inhibitor (5-fluoro-2-deoxyuridine), *de novo* synthesis of dTMP was irreversibly blocked, resulting in a 3,3-4 fold increase over untreated cells in the amount of uracil. This level, although high enough, is still far from the level reached in the *dut-*, *ung-* strain [95].

## Human dUTPase and Drosophila dUTPase: similarities and differences

Most dUTPases, including also the human and Drosophila orthologs, are homotrimer proteins and they contain three identical active sites for dUTP hydrolysis [96]. The structure of dUTPase is evolutionary well-conserved, active sites are located at the subunit interfaces and are comprised of five different conserved motifs of the three monomers [96].

Two isoforms of human dUTPase were identified in human cells, which are encoded by the same gene, but arise from alternative promoter usage and alternative splicing of 5' exons [97]. Thus the two isoforms are largely identical, differing only in a short region of their amino-terminal sequences, which contain distinct localisation signals for both isoforms. Consequently the two isoforms are targeted to different compartments of the cell where DNA synthesis could occur, thus to nucleus and to mitochondria [97]. The one found in the nucleus (DUT-N), contains a nuclear localisation signal [98], and its expression level is upregulated during S phase of cell cycle, during nuclear DNA replication. dUTPase expression was increased in actively dividing cells, while it was hardly detectable in resting cells [97]. In contrast to this, the mitochondrial isoform (DUT-M) is constitutively expressed [97]. Consensus site for cyclin dependent kinase phosphorylation was identified within the amino terminal region of both DUT-N and DUT-M both isoforms, however this posttranslational modification was found to be exclusive for DUT-N *in vivo*, affecting Ser11 residue [99]. *In vitro*, cdc2 kinase is capable to phosphorylate Ser11 suggesting a link to the cyclin signalling pathway [99]. Phosphorylation of NLS or other sequences nearby could regulate the localisation of certain proteins, although such effect of Ser11 phosphorylation for DUT-N was not reported in the literature.

*Drosophila melanogaster* cells also contain two dUTPase isoforms, generated by alternative splicing, but these are both expressed under the control of the same cell-cycle-dependent promoter, and both are therefore present only in actively dividing cells [100]. The two isoforms (21kDa and 23kDa) form homotrimer proteins associated with 63kDa and 69kDa apparent molecular masses respectively, and are termed as long (23kDa) and short (21kDa) isoform. Between these two, only the long (23kDa) contains the complete putative NLS sequence (<sub>10</sub>PAAKKMKID<sub>18</sub>), while the short isoform (21kDa) lacks the N-terminal fourteen residues, thus lacks the PAAKK peptide segment of the putative NLS. The short isoform also lacks any detectable localization signal, and its presence could not be detected in mitochondrial cell fraction [100].

(Figure 13) Surprisingly, localisation studies carried out with ovaries, embryos and larvae; immunohistochemistry and Western blotting of nuclear, cytoplasmic and mitochondrial extracts indicated that both *Drosophila* dUTPase isoforms can be present either in the nucleus or in the cytoplasm in different tissues and developmental stages preventing clear assignment of the two isoforms to distinct dedicated cellular compartments [100].

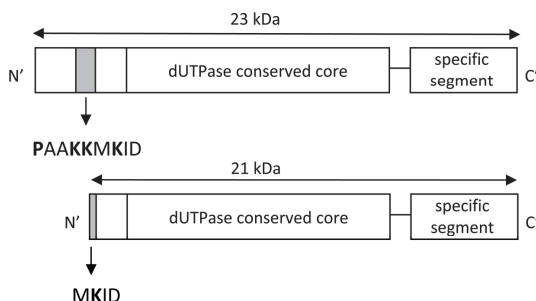


Figure 13 Two isoforms of *Drosophila melanogaster* dUTPase. Both isoforms contain a 28-residue-segment at the C-terminus that is found only in the *Drosophila* dUTPases.

### Nuclear localisation signal sequence of dUTPase

Both, the experimentally verified nuclear localisation signal observed in nuclear isoform of human dUTPase and the putative NLS of *Drosophila* dUTPase N-terminal sequence are rather unusual but show similarity to human c-myc and RanBP nuclear localisation signals [100]. These NLSs represent members of a distinct NLS class, where a short cluster of basic residues is flanked by neutral and acidic amino acids. The *Drosophila* dUTPase NLS also resembles the previously described human dUTPase NLS and human RanBP3 NLS as it contains only 3 positively charged amino acids in the middle of the NLS motif [98, 100].

### Life cycle of *Drosophila melanogaster*

*Drosophila melanogaster* belongs to Holometabola Superorder (Endopterygota) of Insecta Class. Classification of these insects is based on, inter alia, characteristics of their life cycle and development, representing an insect group which develops through holometamorphosis, associated with embryo, larvae, pupae, and imago stages, separated with events of ecdysis.

After the third larval stage, development is continued by puparium formation. In pupae stage, during metamorphosis, larval tissues are degraded and tissues of imago are formed. Breakdown of larval tissues are found to occur via regulated cell death pathways, such as apoptosis and autophagy. Imaginal tissues (discs), group of tissues and cell islets that preserved their differentiation potential during larval stages, serve as a source of adult tissues. An obtrusive difference between larval and imaginal tissues is that DNA in larval tissues goes through cycles of endoreplication, repeated cycles of DNA synthesis without mitosis, while in imaginal cells DNA replication is followed by mitotic cycle. Consequently, endoreplication gives rise to polytene chromosomes, for example giant chromosomes of larval salivary gland, but the genome of imaginal disc preserves its diploid state throughout larval stages. With respect to diploidy, neural cells are similar to imaginal discs.

### **Specific attributes of uracil repair in *Drosophila melanogaster***

*Drosophila melanogaster* represents a unique model organism for uracil-DNA targeted research, because the major and most active uracil-DNA glycosylase, UNG is not encoded within the genome. Therefore UNG activity, which would be responsible for repairing U:A, U:G and uracil in single stranded DNA in both nuclear and mitochondrial DNA is missing. Even though UNG is missing, homolog for SMUG-type uracil-DNA glycosylase protein is encoded, which otherwise was only reported from Vertebrates [66]. TDG and MBD4 homolog sequences could be also identified in the *Drosophila* genome. Substrate preference of Thd1 (*Drosophila* TDG) tends to show a shift towards G:U repair and it is less active on G:T mispairs [71]; however <sup>met</sup>C/C ratio is approximately 50 times lower in *Drosophila* than in mammals indicating that DNA is less intensively methylated in *Drosophila* [2]. Thus G:T mispairs might require less attention of DNA repair pathways.

dUTPase, key factor in preventive uracil-DNA repair, was found to be present with decreased protein level during larval stages, furthermore dUTPase was under detection limit in larval tissues, and was only expressed in imaginal tissues [100]. These recent findings have provided the basic question and working hypothesis for this piece of work.

As seen previously, uracil content of DNA is remarkably increased in dut-ung- *E.coli* cells, due to lack of uracil repair and high level of dUTP incorporation [95]. Similar situation, thus elevated level of uracil in DNA, could be envisioned in *Drosophila* larval tissues. Presence and absence of dUTPase, therefore putative uracil accumulation in *Drosophila* genome could



be associated with cell fate during metamorphosis. Larval tissues lacking dUTPase expression, possibly with higher uracil content, are predestined to death; in contrast to this, dUTPase expressing imaginal tissues, with normal level of uracil survive and develop. Correlation observed between presence of dUTPase and cell fate has raised the possibility that uracil content of DNA may have an impact on development [100]. According to my working hypothesis, uracil accumulated in DNA might be targeted for DNA breakdown during metamorphosis, thus a uracil-DNA degrading mechanism, similar to thymineless cell death, could take part in development of fruitfly supplementing the well-described apoptosis and autophagy pathways.

Absence of UNG from mitochondria is coupled with the absence of mitochondrial dUTPase isoform [100]. In other model organisms, constitutively expressed mitochondrial isoform of UNG is responsible for mitochondrial uracil repair, and mitochondrial dUTPase isoform ensures the correct dUTP/dTTP ratio. In theory, lack of dUTPase in mitochondria would not coexist with presence of highly efficient UNG. Details of mitochondrial uracil-DNA repair are underrepresented in literature for discussion in more depth. As 21kDa isoform of *Drosophila* dUTPase was predicted to stay in the cytoplasm, further investigations were required to describe its role.

## Aims

The present study aimed to investigate aspects of uracil-DNA in *Drosophila melanogaster* and to achieve the followings:

- 1) Verifying the putative nuclear localisation signal, and describe the subcellular localisation of two dUTPase isoforms.
  - Identification of putative NLS segment conserved among dUTPases
  - Cloning of dUTPase-eYFP fusion protein constructs into *Drosophila* Schneider 2 cell line-specific inducible expression vector
  - Creating stable expression system for dUTPase-YFP constructs in Schneider 2 *Drosophila* cell line and follow their distribution within the cell
  - Tracking localisation of dUTPase isoforms in *Drosophila* embryo with special emphasis on mitosis
- 2) Investigate cellular response to uracil-DNA in *Drosophila* cell culture
  - Examining cellular response to uracil substituted plasmid DNA
  - Analysing cellular response to misregulated dUTP/dTTP ratio
- 3) Investigate cellular response to uracil-DNA in fruitfly
  - Examining cellular response to uracil substituted plasmid DNA in embryo
  - Creating an RNAi system for dUTPase in larvae and pupae
  - Describing phenotype of dUTPase silencing
- 4) Measuring uracil content of *Drosophila* biological samples
  - Recording changes of uracil content during development
  - Comparing uracil-DNA level in larval and imaginal tissues
  - Measuring uracil content in response to dUTPase RNAi and misregulation of dUTP/dTTP ratio

## Materials and methods

### 1. Methods used for verifying the putative nuclear localisation signal, and describe the subcellular localisation of two dUTPase isoforms

#### Culturing *Drosophila* Schneider 2 cells

The *Drosophila* Schneider 2 (S2) cell line was derived from a primary culture of late stage (20-24 hours old) *Drosophila melanogaster* embryos [101]. It comprises cells of different origin: epithelial-like cells as well as macrophages are both present.

S2 cells grow at room temperature without CO<sub>2</sub> in a loose, semi-adherent monolayer in tissue culture flasks. If maintained at 25-28°C, they divide once in every 24 hours. It is suggested to pass S2 cells when cell density has reached 5×10<sup>6</sup> cells/ml and split at a 1:2 to 1:5 dilution. Importantly, S2 cells do not grow well when seeded at density below 5×10<sup>5</sup> cells/mL. The culture media used were serum free medium (Gibco) supplemented with 20 mM L-glutamine, 10 U/mL penicillin, 0.1 mg/mL streptomycin (Sigma).

#### Cloning of dUTPase-eYFP fusion protein constructs

pRmNDUT-eYFP (21 kDa dUTPase) and pRmDUT-eYFP (23 kDa dUTPase) vectors were constructed by cloning 21 kDa and 23 kDa dUTPase coding sequences into the *Drosophila* transfection vector pRm-eYFP-N-C\* [102]. The pRmDUTeYFP and pRmNDUT-eYFP constructs produce dUTPase with a C-terminal eYFP (enhanced yellow fluorescent protein) under the control of a metallothionein promoter. The metallothionein promoter contains a heavy metal shock response element, therefore administration of heavy metal ion, in our case Cu<sup>2+</sup> switch on the expression of the encoded protein.

The LDdut-pET22b plasmid was used as a template for amplification of *D. mel.* dUTPase gene by PCR. The PCR was performed with two different forward primers and a single reverse primer (Table 6). These primers introduce a NheI site upstream and a NotI site downstream of the gene, which was used for directional insertion into the target vector. The sequences of plasmids were verified by sequencing. (MWG-operon, Germany)

Forward primers	5'-ctagctagcatgccatcaaccgatttcgccgacattc-3'	23kDa	NheI
	5'-ctagctagcatgaagatcgacacgtgcgtcctgcg-3'	21kDa	NheI
Reverse primer	5'-atagtttagcggccgctagcaacaggagccggagc-3'	both	NotI

Table 6 Primers used for cloning of 23kDa and 21kDa dUTPase isoform into pRm-eYFP-N-C\*

### **S2 cell culturing, transfection and selection**

For stable transfection of S2 cells, dUTPase-eYFP expression constructs were cotransfected with pPURO plasmid in 1:20 molar ratio. pPURO plasmid encodes puromycin resistance gene. Transfection was carried out in the presence of Cellfectine. Stable cell lines were selected by growing them in the presence of increased concentration of puromycin. The final puromycin concentration was 50µg/mL.

### **Localisation of dUTPase-YFP in S2 cells**

For investigating the subcellular distribution of dUTPase isoforms, dUTPase-eYFP, DNA and actin were visualized. Selected S2 cells were cultured on microscope slides. Expression of dUTPase-eYFP constructs was induced at 25°C by addition of 700 µM CuSO<sub>4</sub> and overnight incubation. Cells were washed with PBS, fixed in 3% paraformaldehyde for 5 minutes, and permeabilized with 0.1% Triton-X 100. DNA was stained with DAPI (Sigma) and actin was labeled with rhodamine –phalloidine. Samples mounted in FluorSave Reagent (Calbiochem) were visualized with Olympus IX70 confocal laser scanning microscope, under a 60× oil immersion objective.

### **Microinjection of S2 cell extract into Drosophila embryo, confocal microscopy**

Localisation of dUTPase throughout complete cell cycle was investigated in Drosophila embryos. Drosophila embryos in the syncytial blastoderm stage (app. 2h after egg laying, cycles 10-13) offer a particularly useful model to study mitosis, because in this stage mitotic events are strongly synchronized within the embryo and nuclei are large enough for convenient visualisation.

S2 cell extract, prepared from cells expressing dUTPase-eYFP fusion proteins, was injected into Drosophila embryos for these localisation studies.

After overnight induction of dUTPase-eYFP expression, S2 cells were washed off by pipetting and washed in PBS. Cell pellet was homogenized on ice in equal volume of lysis

buffer (150 mM NaCl, 10% glicerol, 10 mM Tris pH 7.4, 1 mM DTT, protease inhibitor), then lysate was cleared by centrifugation (14300 rpm, 15 min, 4°C). Rhodamine-tubulin was added to the S2 cell extract in order to follow dynamics of microtubule assembly related to nuclear divisions. Approximately 200 pL of freshly prepared cell extract supplemented with rhodamine-tubulin (2% of total egg volume) was injected into the posterior region of wild-type (Oregon R) embryos at syncytial blastoderm stage (cycles 10-13). Embryos were dechorionated in 3% sodium hypochlorite before injection. Localisation of the two dUTPase isoforms were followed separately over time by series of optical sections generated with an Olympus VS1000 confocal microscope. The injections were carried out at 20°C. Localisation studies in *Drosophila* embryos were done in collaboration with Prof. János Szabad and Zsolt Venkei in Szeged.

## **2. Methods used for investigating cellular response to uracil-DNA in *Drosophila* cell culture**

### **Culturing *Drosophila* Schneider 2 cells** (described previously)

Here, the culture media used were Serum Free Insect Medium (Sigma, S3777) supplemented with 10 Unit/mL penicillin and 0.1 mg/mL streptomycin (Sigma).

### **Culturing human HeLa cells**

Human HeLa cell line was propagated from cervical cancer cells. It grows well in 5% CO<sub>2</sub> at 37°C. For experiments described here, HeLa cell was maintained in DMEM-F12 culture media (Sigma, D8437) supplemented with 10% FBS and 1% penicillin–streptomycin solution. Studies on HeLa cell culture were carried out by András Horváth and Gábor Merényi.

### **Alamar blue assay for determining the effect of 5'-fluorouracil and 5'-fluorodeoxyuridine**

The drug 5'-fluorouracil (5'FU) and 5'-fluorodeoxyuridine (FUDR) are frequently used as an inhibitor of thymidylate metabolism. Treatment leads to perturbation of nucleotide levels and induces elevated level of dUTP, thus major increase in the uracil content within DNA. Effect of 5'FU and FUDR on S2 cells therefore indicates cellular response given to elevated level of uracil in genomic DNA in *Drosophila* cells. For comparison, treatment was also applied to mammalian cells.

Alamar blue is widely used reagent for the analysis of cell proliferation and cytotoxicity in response to certain drug treatment. It gives an indirect measure of cell viability by exploiting the natural reducing power of living cells to convert resazurin to resorufin. Resazurin is a cell permeable reagent that is blue in color and nonfluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby the amount of fluorescence produced is proportional to the number of living cells.

Drosophila Schneider 2 (S2) cells and human HeLa cells were cultured in 96 well plates at  $5 \times 10^4$  cells/well or  $2 \times 10^3$  cells/well, respectively, with appropriate drug treatment in a final volume of 100  $\mu$ l/well. 5'-fluorouracil (Sigma) and 5'-fluorodeoxyuridine (FUdR) was added at a final concentration range of 0.1–1000  $\mu$ M. After 96 hours in culture, cell viability was quantified by Alamar Blue assay (BioSource). Assay was performed according to the manufacturer's protocol: Alamar Blue solution was directly added to the medium in a final concentration of 10 %, followed by a 4 hours period of incubation, then absorbance was read at 540 nm. The number of viable cells correlates with the magnitude of dye reduction. Data was normalized with the absorbance of control, lacking 5'-fluorouracil treatment cells. The experiment was repeated in triplicates.

### **U-plasmid interpretation assay in cell culture**

Uracil-DNA specific cell response was provoked by transfecting cells with exogenous plasmid uracil-DNA in order to decide whether such chemically unusual DNA may be tolerated and interpreted in Drosophila cells.

Fluorescent protein encoding plasmids, pRm-eYFP-N-C\* for S2 cells, pDsRed-Monomer-N1 (Clontech) for HeLa cells were amplified in *E.coli* K12 XL1Blue strain and CJ236 ung-, dut-strain (NEB), producing normal DNA plasmid and dUMP-substituted version of the construct (U-plasmid) respectively. Plasmids were purified with Plasmid Midi Kit (QIAGEN). Uracil content of the plasmids was checked with UDG and AP endonuclease treatment followed by standard agarose gel electrophoresis.

Transfection of pRm-eYFP-N-C\* into Drosophila Schneider 2 cells was carried out in the presence of Cellfectine (Invitrogen) and expression was induced from the metallothionein promoter by addition of 700 $\mu$ M CuSO<sub>4</sub> and overnight incubation. pDsRed-Monomer-N1 was

transfected into HeLa cells by using Lipofectamine (Invitrogen). Samples were visualized with a Leica DMLS fluorescence microscope.

Barbara Hodoscsek undergraduate student also contributed to this work.

### 3. Methods used for investigating cellular response to uracil-DNA in fruitfly

#### U-plasmid interpretation assay in *Drosophila* embryo

Uracil-DNA plasmid interpretation assay was carried out in *Drosophila* embryo similarly to the cell culture reporter assay. These experiments were made in collaboration with Miklós Erdélyi, BRC, Szeged.

Uracil-DNA plasmid was introduced to *Drosophila* embryo by microinjection. Three different experimental setups were tried out, differing in plasmid DNA and *Drosophila* strain. Table 7 summarises the plasmid-*Drosophila* pairs.

<b>Drosophila strain (embryo)</b>	<b>Plasmid</b>
nosGal4 transgene	UASp-GFP
wild type	Act-GFP
UASp-GFP transgene	Promoter-Gal4VP16

Table 7

Plasmids were amplified in *E.coli* K12 XL1Blue strain and CJ236 ung-, dut- strain (NEB), and purified with Plasmid Midi Kit (QIAGEN).

For each experiment, app. 40 0-30 min dechorionated embryos were microinjected. They were aligned on a glass coverslip, dried for 30 min, than covered with 10S Voltalef oil before injection. Plasmid concentration was adjusted to 1mg/mL, by dilution in standard injection buffer.

GFP signal would have indicated the interpretation of exogenous DNA. GFP signal was detected in pre-hatching embryos, 22h after injection.

### Gal4/UAS system

Gal4/UAS system is widely used for addressing gene function in vivo and for its flexible application it was called as the “Fly Geneticist’s Swiss Army Knife”. It allows ectopic gene expression in a wide variety of cell- and tissue-specific patterns in *Drosophila* [103].

It consists of two components: Gal4 and UAS (Figure 14). A promoter drives the expression of Gal4 yeast transcriptional activator and a Gal4-responsive UAS (Upstream Activating Sequences) enhancer element directs the expression of gene of interest. Initially, the two components are encoded in two different transgenic fruitfly lines. In the Gal4 line, also called as driver, Gal4 has no target gene to activate. In the UAS-line, the target gene is silent because of lack of activator Gal4. It is only when the Gal4 line is crossed to the UAS–target gene line that the target gene is turned on in the progeny and it is expressed under the spatial and temporal control of Gal4’s promoter. Depending on the driver, effect of a variety of expression pattern can be tested [103].

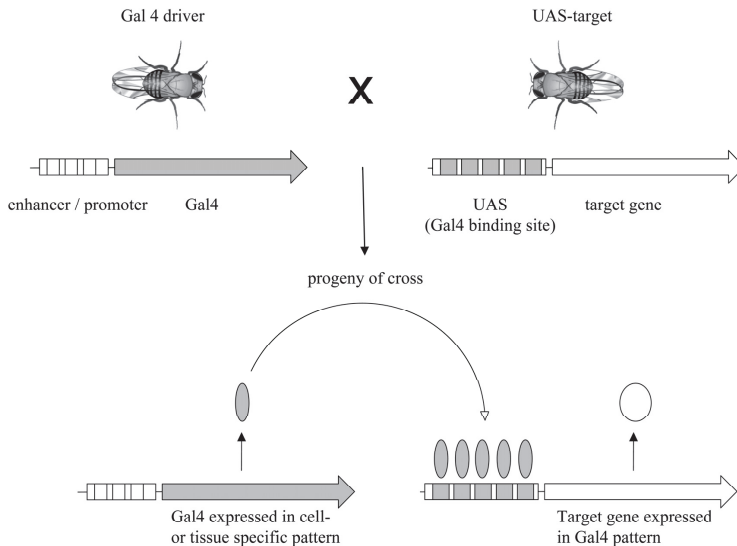


Figure 14 Gal4/UAS expression system in *Drosophila*. Figure was reproduced after Brand *et al.* with minor modifications [104].



## RNA interference

In general RNA interference (RNAi) leads to silencing of a certain gene by reducing its mRNA level available for translation. In *Drosophila melanogaster*, RNAi can be induced with long double stranded RNA, typically 300-400bp in length. Contrary to mammalian systems, short interfering RNAs (siRNA), 21-23bp, are not efficient in *Drosophila*.

Transgenic *Drosophila melanogaster* strains for dUTPase silencing were purchased from Vienna *Drosophila* RNAi Center (VDRC). Each strain contains an inducible UAS-RNAi construct against the dUTPase. The generic GAL4/UAS system is used to drive the expression of a hairpin RNA (hpRNAs), which are encoded as inverted repeat sequences (IR) [105]. The emerging double-stranded RNAs are processed by Dicer into siRNAs which direct sequence-specific degradation of the target mRNA. By using different Gal4 drivers, effect of silencing could be followed separately in different tissue and cell types as well as in different developmental stages.

RNAi was induced against dUTPase mRNA by using Act5C-Gal4 /Cyo, GFP GMR-Gal4 and engrailed-Gal4/CyO, GFP drivers. Act5C-Gal4 causes ubiquitous expression of GAL4. Drivers GMR-Gal4 and engrailed-Gal4/CyO are imaginal disc-specific, active in eye and wing discs respectively.

Silencing was induced by crossing UAS-IR males to virgin females carrying adequate drivers. Progeny were raised at 25°C. For Act5C-Gal4 /Cyo, GFP, they were scored according to GFP marker in larvae or Cyo wing marker in imago. Animals lacking GFP and Cyo wing markers express the IR construct thus silence dUTPase (Figure 15). Reduced number of hatched non-Cyo imago as compared to the number of Cyo imago indicates the importance of silenced gene for development. Progeny of GMR-Gal4 x UAS-IR and engrailed-Gal4/CyO x UAS-IR crossings were subjected to careful observation with stereo microscope.

In order to test the effect of ubiquitous Gal4 expression, wild type ( $w^{1118}$ ) males were also crossed to ActGal4/Cyo, GFP virgins and segregation ratio was assessed.

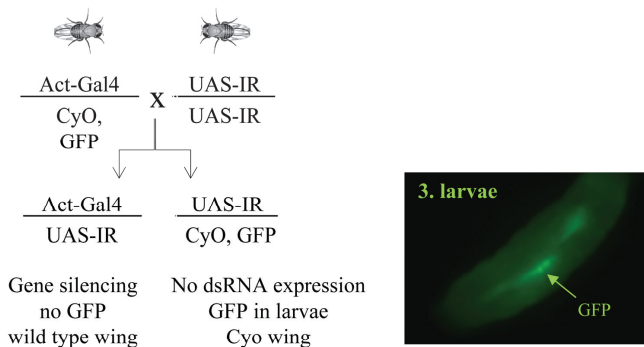


Figure 15 dsRNA expressing animals are distinguishable at larvae and imago stages on the basis of GFP and CyO markers

For #21883 and #21884 *Drosophila* strains, insertion site of the P-element, containing the UAS-IR sequence, was determined by inverse PCR and sequencing according to BDGP (Berkeley *Drosophila* Genome Project) protocol. Data would further confirm that the observed phenotype is truly a consequence of dUTPase silencing and would exclude the possibility that change in expression of UAS-IR insertion neighbouring genes would have caused it.

Genomic DNA prepared from ~15 adult flies, was digested with MspI restriction enzyme and fragments were circularised in a ligation reaction. For inverse PCR, three different primer pair combinations were tried out: /Plac1, Pwht1/, /Pry1, Pry4/ and /Pry1, Pry2/ which amplified 5' or 3' flanks of insertion. PCR products were purified from agarose gel with QIAquick Gel Extraction Kit (QIAGEN) and sent for sequencing (MWG-Operon).

Name	Sequence	Application
Plac1	CACCCAAGGCTCTGCTCCCACAAT	inverse PCR
Pry1	CCTTAGCATGTCCGTGGGGTTTGAT	inverse PCR
Pry2	CTTGCCGACGGGACCACCTTATGTTATT	inverse PCR, sequencing
Pry4	CAATCATATCGCTGTCTCACTCA	inverse PCR
Pwht1	GTAACGCTAATCACTCCGAACAGGTCACA	inverse PCR
3.SUP.seq1	TATCGCTGTCTCACTCAG	sequencing
5.SUP.seq1	TCCAGTCACAGCTTTGCAGC	sequencing

Table 8: Primers used for determining sequences flanking UAS-IR (DUT) insertions

Stock #	Source	Description
#21883	VDRC	dUTPase RNAi, UAS- dut IR expression
#21884	VDRC	dUTPase RNAi, UAS- dut IR expression
-	BRC	actin-Gal4/CyO, ubiquitous driver
-	lab	actin-Gal4/CyO GFP, ubiquitous driver
-	BRC	GMR-Gal4, eye disc specific driver
-	BRC	engrailed-Gal4/CyO, GFP, wing disc specific driver
OregonR	BRC	wild type
w <sup>1118</sup>	BRC	white eye, wild type

Table 9. *D.melanogaster* stocks for dUTPase RNAi

Efficiency of silencing was determined by performing western blot with anti-dUTPase polyclonal antibody on 3<sup>rd</sup> stage larvae and pupae samples.

### Western blot

Extracts were run on 12% SDS-PAGE and transferred to PVDF membrane in 10mM CAPS, 10% methanol transfer buffer. Blots were stained first with Ponceau protein dye and then developed with anti-dUTPase polyclonal antiserum (1:100000 dilution) and anti-rabbit IgG-HRP secondary antibody (1:2,500 dilutions). For visualization, enhanced chemiluminescence reagent of Amersham Biosciences was used. Monoclonal anti-actin antibody (Sigma) in was used as loading control.

### Creating ActGal4/CyO,GFP *Drosophila melanogaster* stock

GFP marker was essential to discriminate silenced and non-silenced progeny during larval stages. For obtaining an actin-Gal4/CyO GFP stock, balancer chromosome of actin-Gal4/CyO was changed to CyO GFP second chromosomal balancer chromosome. This was achieved by two crosses: first, engrailed-Gal4/CyO, GFP x w<sup>1118</sup> generates +/CyO, GFP progeny which has white eye and curly wing phenotype, second +/CyO,GFP x actin-Gal4/CyO generates actin-Gal4/CyO GFP (red eye and curly wing)

### Maintaining *Drosophila melanogaster*

*D.melanogaster* was cultured at 25°C or 18°C on cornmeal, dextrose and yeast medium.

#### **4. Method used for measuring uracil content of *Drosophila* biological samples**

In order to quantify uracil content of DNA, a real-time quantitative PCR-based assay was developed recently by my colleague, András Horváth, Laboratory of Genome Metabolism and Repair. It measures the amount of uracil within a selected DNA sequence. The sequence-specific manner of this technique is unique among other methods developed for quantifying uracil content.

The technique is based on two separate quantitative PCR reactions where two different types of DNA polymerases are used. The wild type enzyme Pfu from *Pyrococcus furiosus* (species of Archaea) selectively accepts uracil-free DNA template only. If Pfu detects uracil in the template strand, it stops the DNA synthesis. This enzyme is used to determine the amount of uracil-free template. A variant of Pfu DNA polymerase, where the uracil binding pocket was mutated, is not sensitive to uracil; therefore it is capable to amplify all DNA templates. The amount of uracil containing template can be calculated from the number of uracil free-DNA template and from the number of the total amount of DNA template. The amount of uracil containing template, and the length of template sequence can determine the chance for a single nucleotide being uracil, if an assumption is taken that there is only one uracil in every uracil containing template.

In all measurements, uracil content of a DNA sample is correlated to a reference DNA sample which is considered to be uracil-free. For most measurements plasmid DNA or genomic DNA from *Drosophila* embryo was used.

##### **DNA preparation**

Genomic DNA was isolated by using MasterPure DNA Purification Kit (Epicentre). DNA was subsequently digested with NheI (New England Biolabs) restriction enzyme at 37°C, overnight. DNA was run on 1% agarose gel electrophoresis and fragments of 4000-5000 nucleotides in size were purified from gel with QIAquick Kit (Qiagen). Fragmentation and size selective fragment purification enriches the targeted DNA sequence within the DNA sample.

## qPCR

Real-time PCR reactions were performed in Mx3000P qPCR System (Agilent Technologies, Stratagene) in plates, 96-well format using EvaGreen dye (Biotium) to monitor amplification. In our experimental setup, a segment with 963 base length defined by the primers (puBSd985R and puBSd-Fw) is amplified during the PCR reaction.

Two-fold dilution series were prepared from DNA samples. Instead of working in triplicates, each step of serial dilution served as parallel measurement. For all samples, reaction mixture with PfuTurbo Hotstart DNA polymerase (uracil-sensitive) (Stratagene) and with Pfu Turbo Cx Hotstart DNA polymerase (uracil-nonsensitive) (Stratagene) was set up. (Table 10)

1 µl	DNA
1 µl	PfuTurbo Hotstart DNA polymerase buffer 10x
0,5 µl	EvaGreen 50x
0,2 µl	dNTPmix (10mM) (Fermentas)
0,175 µl	forward primer 10 pmol/µl
0,175 µl	reverse primer 10 pmol/µl
0,01 µl	ROX (passive reference dye)
6,75 µl	nuclease free water (Ambion)
0,2 µl	PfuTurbo Hotstart DNA polymerase (wt/Cx)

1x	2 min	95 °C
40x	15 s	95 °C
	10 s	57 °C
	70 s	72 °C

name	sequence	template
puBSd-Fw	5' TCGGGATGACTTTTGGGTTCTG3'	chromosome 2L, 11010370...11010391
puBSd985R	5' CGCGGTTTAACACAGCGTCGG3'	chromosome 2L, 11011354...11011334

Table 10 Reaction mixture, primer sequences and thermal cycle for uracil content measurement

## Calculation

Uracil content of DNA sample is calculated from the measured Ct (threshold cycle) values of two reactions: reaction with wild type Pfu (detects uracil-free DNA) and with Cx-type Pfu (detects all DNA).

For each step of serial dilution, the measured Ct values of wild type Pfu reaction are plotted in function of Cx-type Pfu Ct values, and linear fitting is applied (Figure 16). The wild type Pfu

polymerase can not amplify uracil-containing DNA; therefore it will give higher Ct values. Difference between the two linear curves is proportional to the amount of uracil present in DNA.

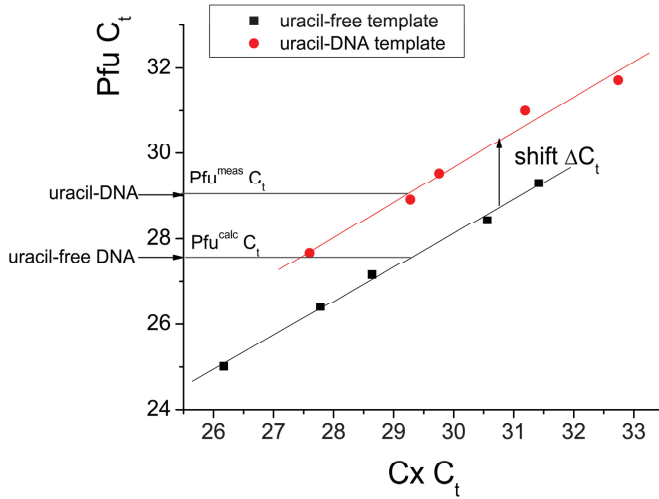


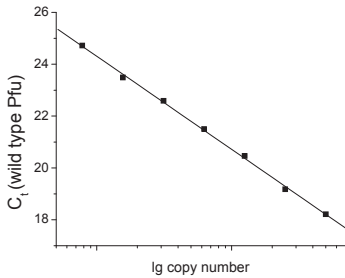
Figure 16 There is a shift between linear slopes of uracil-containing DNA sample (red points) and presumably uracil-free DNA sample.

In the next step,  $\Delta C_t$  values are calculated. The equation of uracil-free linear curve is used to determine the calculated  $Pfu C_t$  values ( $Pfu^{calc} C_t$ ), which would have been measured if the DNA had been uracil-free.

Then,

$$Pfu^{meas} C_t - Pfu^{calc} C_t = \Delta C_t$$

Next, value of amplification efficiency of polymerase reaction is determined according to the equation below, where M is the slope of the efficiency curve (Pfu Ct values are plotted in function of copy number in logarithmic scale) and A is calculated from M. Figure 17



$$10^{\frac{-1}{M}} = A$$

Figure 17 Efficiency curve. Ct values measured with wild type Pfu were plotted in function of copy number and represented in logarithmic scale

Uracil content is then calculated

$$1 - \left[ A^{-\Delta Ct} \right]^{\frac{1}{963}} \times 100 \rightarrow \%Uracil$$

Value of 963 comes from the length of the measured sequence.

Some of the measurements that I present in my thesis were carried out by András Horváth.

## Results

### Subcellular localisation of *Drosophila* dUTPases

#### Identification of a putative NLS segment conserved among dUTPases

The putative NLS sequence of *Drosophila* 23 kDa dUTPase shows homology with the NLS of human c-myc and RanBP3 (Table 11A). Comparison of dUTPase sequences from different eukaryotic species has revealed that they possess similar NLS segments at their N termini as found in *Drosophila* or human larger isoform (Table 11B). In spite of the fact, that most classical NLS contains at least 5 basic amino acids within a nonapeptide, these NLSs consists of a short cluster of basic residues flanked by neutral and acidic amino acids. The *Drosophila* dUTPase NLS also contains only 3 positively charged amino acids in the middle of the NLS motif. It indicates that dUTPase NLS belongs to a rather unusual type of NLS class.

The PAAKKMKID sequence motif is 100% conserved among available *Drosophila* genomes with exceptions of *D. willistoni*, *D. virilis* and *D. grimshawi* (Table 11C).

A		B	
<b>D. melanogaster dUTPase</b>	PAA <b>KKMK</b> ID	<b>D. melanogaster dUTPase</b>	PAA <b>KKMK</b> ID
H.sapiens c-myc	PAA <b>KRVK</b> LD	H. sapiens dUTPase	SPS <b>KRAR</b> PA
H.sapiens dUTPase	SPS <b>KRAR</b> PA	M. musculus dUTPase	SAS <b>KRAR</b> AE
H.sapiens RanBP3	PPV <b>KRERT</b> S	R. norvegicus dUTPase	SVS <b>KRAR</b> AE
C		G. gallus dUTPase	SPS <b>KRK</b> KGS
<b>D. melanogaster dUTPase</b>	PAA <b>KKMK</b> ID	C. elegans dUTPase	PAL <b>KK</b> S <b>KTE</b>
D.willistoni dUTPase	QA <b>AKMK</b> LE	L. esculentum dUTPase	PSP <b>KVQK</b> LD
D.grimshawi dUTPase	QSA <b>KKIK</b> MD	O. sativa dUTPase	PLL <b>KVKK</b> LS
D.virilis dUTPase	QT <b>AKMK</b> LD	Consensus	
		P <b>AzKKxKz</b>	
		S <b>PzKRxRz</b>	

Table 11. dUTPases possess an unusual NLS.

A | *Drosophila* dUTPase NLS is similar to human c-myc, RanBP3 and human dUTPase NLS sequences. B | Examined dUTPases from different eukaryotic organisms possess similar NLS as found in *Drosophila* or human larger isoforms. These NLS sequences contain only 3 basic amino acids at conserved positions, and in many of them, one proline residue is also present. z: non-charged amino acids., x: any residue C | Variance of NLS sequences among *Drosophila* species. Positively charged amino acid residues are highlighted in bold.



## Localisation of *Drosophila* dUTPase isoforms in S2 cells

Even though transfection of dUTPase-eYFP construct into S2 cell by using Cellfectin reagent did not allow immediate observation because of its low efficiency, continuous selection with puromycin resulted in approx 70% of the cells overexpressing the fluorescent construct.

S2 cells overexpressing fluorescently labelled constructs of the 23 and 21 kDa dUTPase isoforms (green) were also stained for DNA (blue) and actin (red), to aid interpretation of the results (Figure 18). The 21 kDa isoform is present around the nucleus and within the cytoplasm in all cells; it is, however, strictly excluded from the nuclei. In contrast to this, in S2 cells, the 23 kDa construct showed nuclear localisation. This observation directly suggests that the N-terminal extension may contain a NLS segment: on one hand, lack of it prevents nuclear import of dUTPase; on the other hand, the 23 kDa isoform with the N-terminal extension is visualized within the karyoplasm (Figure 18).

Immunofluorescent micrographs for 23kDa dUTPase isoform indicate that the distribution of 23kDa dUTPase is not uniform within the nucleus: it is likely to be present in the karyoplasm and it might be excluded from the nucleolus.

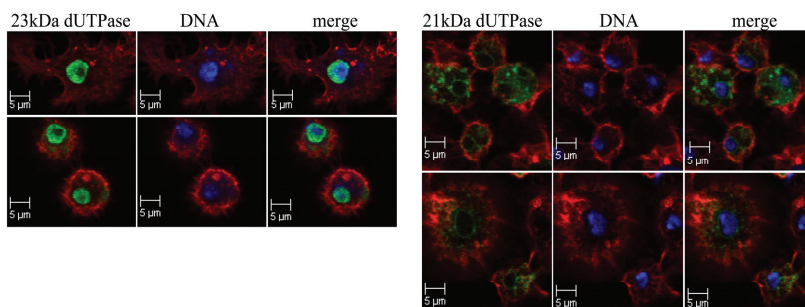


Figure 18 Localisation of *Drosophila* dUTPase isoforms in S2 cells. The 23 kDa isoform shows nuclear localization, while the 21 kDa isoform is cytoplasmic.

### **Localisation shifts of *Drosophila* dUTPase within embryos**

S2 cell extract, containing the 23 kDa or 21 kDa fluorescent dUTPase constructs (green), was microinjected into embryos and the localisation patterns were followed during nuclear cleavage. Rhodamine-tubulin (red) was also coinjected to aid visualization of the mitotic stages. Steps of mitosis were determined by following the formation and movements of mitotic spindle and centromeres, thus localisation of dUTPase isoforms could have been timed to mitotic stages.

Figure 19 clearly indicates that during interphase, the 23 kDa isoform staining is located within the nuclear space while the 21 kDa isoform is diffusely scattered and is excluded from the nuclei. In agreement with S2 cell culture studies, presence or absence of NLS clearly distinguishes the two isoforms regarding their localisation.

As shown on Figure 19, as nuclei enter mitosis, the two dUTPase isoforms show opposite localisation shifts. Unexpectedly, the 21kDa dUTPase shows a localisation shift to the karyoplasm, meanwhile the 23kDa dUTPase starts to diffuse from the nuclear space. None of the isoforms shows colocalisation with the condensated chromosomes. Later on during metaphase, the 21kDa dUTPase remains around the chromosomes which have aligned at the metaphase plate. Remnants of 23kDa dUTPase can be detected in dividing nuclei, but most of the 23kDa dUTPase is scattered all around in the cytoplasm. Localisation of dUTPase isoforms does not change much during anaphase; the 21kDa dUTPase traces out the chromosomes, which are pulled apart towards the opposite poles. The 21kDa dUTPase gets excluded from the karyoplasm only during telophase, when new nuclei of the daughter cells and their nuclear envelope emerge. By the end of cytokinesis, embryo regains the state of interphase, when 23kDa is nuclear and 21kDa dUTPase is cytoplasmic.

In *Drosophila* embryos during nuclear cleavages, the 23 kDa isoform showed the expected localisation shifts. Contrariwise, although the 21 kDa isoform was excluded from the nuclei during interphase, it was shifted to the nucleus during prophase and forthcoming mitotic steps. The observed dynamic localisation character showed strict timing to the nuclear cleavage phases.

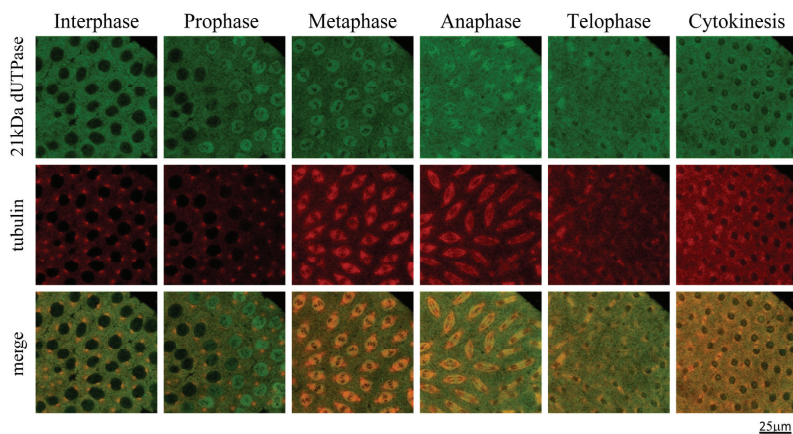
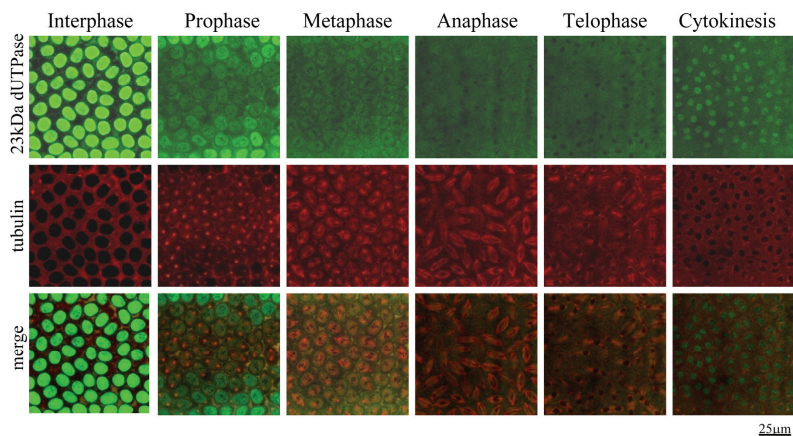


Figure 19 Localization shifts of the two isoforms during the cell division cycle from interphase to cytokinesis. Note the opposing shifts of the two isoforms, evident from prophase.

**Uracil-DNA in *Drosophila melanogaster*: interpretation and developmental involvement**

**Cellular response to uracil substituted plasmid DNA in cell culture**

*Drosophila* S2 cells were transfected with yellow and human HeLa cells with red fluorescent protein by using plasmids pRmYFP and pDsRed-Monomer, respectively. Upon transfection of both human and *Drosophila* cells with normal DNA plasmid, the expression of the fluorescent protein encoded by the plasmid can be readily detected, according to the usual expectation. However, clear-cut difference could be observed upon transfection with uracil substituted plasmid (U-plasmid). Human cells transfected with U-plasmid show hardly any fluorescent signal (only 2-3% of cells) indicating that uracil-substituted DNA was not interpreted, probably due to its degradation. In *Drosophila* cells, however, transfection with U-plasmid leads to expression of the fluorescent protein at levels comparable to that observed in cells transfected with normal plasmid. (Figure 20-21)

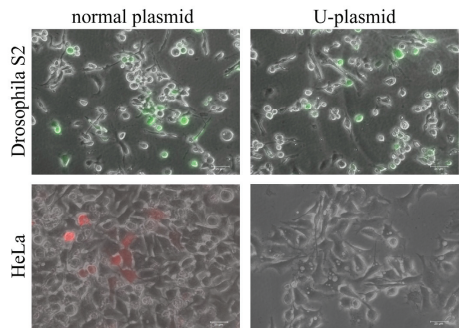


Figure 20 Uracil-plasmid interpretation assays in cell cultures.

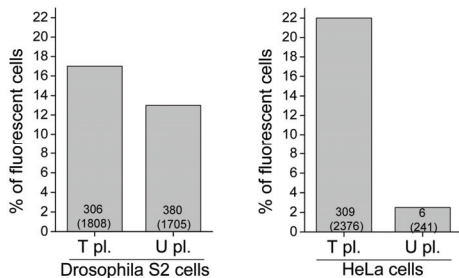


Figure 21 Percentage of fluorescent cells upon transfection with normal (T pl.) or uracil-substituted plasmids (U pl.) The number of observed fluorescent cells is also presented, and the total number of scored cells is shown in brackets.

### Cellular response to misregulated dUTP/dTTP ratio

Drugs 5'-fluorouracil (5'FU) and 5'-fluorodeoxyuridine (FdUR) are inhibitors of thymidylate synthase. Therefore treatment with 5'FU or FdUR would lead to perturbation of nucleotide levels thereby inducing major increase in the uracil content within DNA [106].

Figure 22 reports that cellular responses given to 5'FU and FdUR treatment are different in human and *Drosophila* cells. Both drugs destroy human cells, while *Drosophila* tolerates these drugs very well. In HeLa, the  $IC_{50}$  value for 5'FU was given  $21\mu M$ , which is in agreement with the previously determined  $IC_{50}$  values in mammalian cell lines (Table 22).

These results support that *Drosophila* cells might tolerate elevated level of dUTP incorporation into DNA.

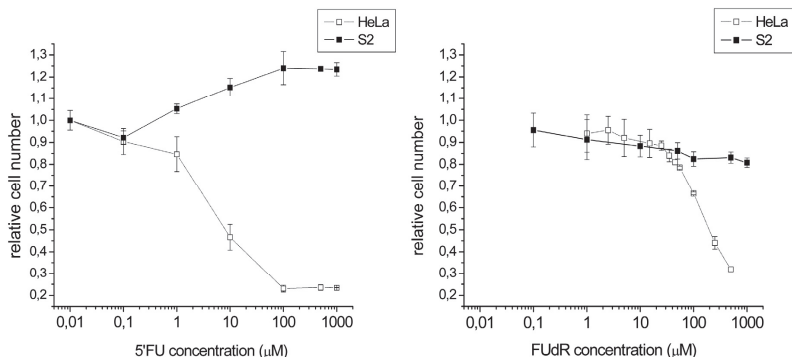


Figure 22 Dose-response curves of 5'FU and FdUR treatments

Cell line	Origin	$IC_{50}$ for 5'FU	Reference
mES	mouse embryonic stem cell	$0.72\mu M$	[107]
C166	mouse yolk sac, endothelial	$1.04\mu M$	[107]
H630	human colorectal cancer	$1\mu M$	[108]
LS174T	human colon cancer	$45\mu M$	[109]
LiM6	human colon cancer	$38\mu M$	[109]
DU145	human prostate cancer	$204\mu M$	[110]
HeLa	human cervical cancer	$278\mu M$	[110]

Table 12. Examples of half maximal inhibitory concentration ( $IC_{50}$ ) of 5'-fluorouracil (5'FU) reported in the literature, determined in different mammalian cell culture assays.

### Examining cellular response to uracil substituted plasmid DNA in embryo

Fluorescent signal was only detectable upon microinjection of Gal4-VP16 expression vector into UAS-eGFP transgenic animals.

Upon microinjection of Gal4-VP16 expression vector into UAS-eGFP transgenic animals, GFP signal indicated the interpretation of exogenous DNA. In pre-hatching embryos GFP signal was detected from both, normal and uracil substituted DNA (Figure 23). In both cases the expression pattern of the reporter construct is identical. It is pronounced in embryonic yolk cells therefore GFP is visible in the gut.

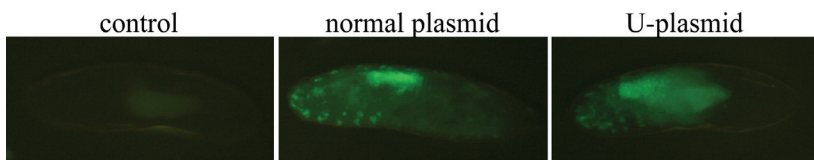


Figure 23 Uracil-plasmid interpretation assays in *Drosophila* embryo

### dUTPase RNAi in *Drosophila melanogaster*

To investigate physiological response given to uracil-DNA in later stages of development dUTPase silencing was induced. Reduced level of dUTPase would lead to dUTP misincorporation into DNA.

Crosses targeting tissue specific silencing of dUTPase, crosses with engrailed-Gal4/Cyo, GFP and GMR-Gal4 drivers, did not resulted in observable eye or wing phenotype.

Ubiquitous silencing of dUTPase was induced by using ActGal4/CyoGFP driver. Under normal physiological circumstances in early *Drosophila* embryo, dUTPase protein and RNA is maternally provided, therefore efficient silencing could be achieved only in later stages of development.

Upon silencing at 25°C, protein level of dUTPase has been reduced below detection limit in 3<sup>rd</sup> larvae and early pupae (Figure 24). In larvae dUTPase is expressed only in imaginal tissues, thus decreased level of dUTPase is due to its clearance from imaginal discs and imaginal cells.

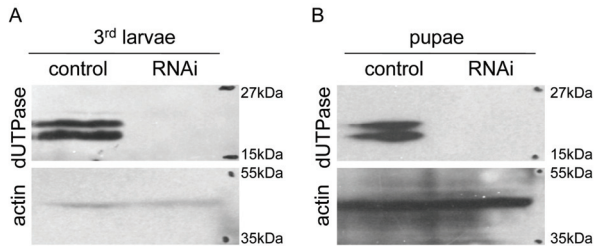


Figure 24 Robust silencing of dUTPase in 3<sup>rd</sup> larvae (A) and pupae (B)

Virtually dUTPase-free larvae did not show any adverse effects indicating that dUTPase silencing did not perturb normal life and development in the larval stages.

At early pupal stage, however, dUTPase silencing led to lethality – no silenced animals could develop into imago and only fruitflies with curly wing, i.e. where silencing does not take place, emerged from pupa. Morphological observations indicated serious adverse effects: failure of head eversion and developmental arrest (Figure 25) [111]. On Figure, arrow points to the developing head, this forms as the imaginal head sac is everted. Upon dUTPase silencing head could not formulate, thus transition from cryptocephalic pupa to phanerocephalic pupa is prevented. In addition to failure to complete this process, development is arrested. By comparing animals 24 hours later, lack of malpighian tubules can be observed. All pupae perish at this stage of development if dUTPase RNAi was induced.

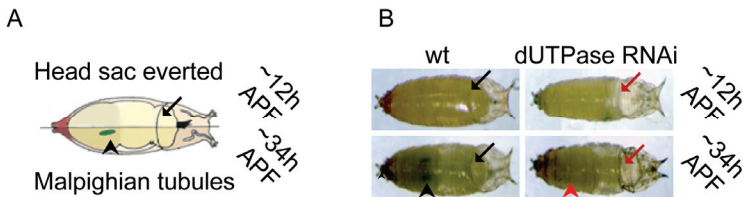


Figure 25 dUTPase silencing has caused failure of head eversion and developmental arrest. Images were recorded approx. 12h and 34h after puparium formation (APF)

The observed adverse effect of dUTPase silencing indicates that lack of dUTPase can be tolerated only at certain stages of fruitfly development. Moreover, it argues that the role of dUTPase in nucleotide metabolism and synthesis is not dispensable

dUTPase silencing was induced with two UAS-IR stocks, #21883 and #21884, and resulted in consistent effects. In both stock, UAS-IR insertion is present on the second chromosome of *Drosophila*, although at different genomic position. Mapping of UAS-IR construct revealed their precise location (Table 13). These data reinforce the conclusion that the observed phenotype is truly due to dUTPase silencing.

VDRC stock#	Insertion site		3' flanking gene	5' flanking gene
21883	2R, 60A	19812632	CG5594	CG2812
21884	2L, 35A	14234587	CG4551	-

Table 13 Genomic position of dUTPase UAS-IR constructs

### Uracil content of *Drosophila* biological samples

In order to test our working hypothesis that uracil could accumulate in DNA of fruitfly larvae; measurement of uracil level was carried out by applying real-time quantitative PCR based method.

Data argues that DNA purified from *Drosophila* embryo is relatively uracil-free and therefore genomic DNA of embryo could be used as a reference DNA sample in later measurements (Figure 26A).

Uracil level was estimated from different stages of development, with high emphasis on larval stages (Figure 26B). Results show that during larval stages uracil is accumulated, and reaches its maximum level at 3<sup>rd</sup> stage larvae. This finding provides clear-cut evidence arguing for the main working hypothesis of this project, that lack of dUTPase and lack of UNG are sufficient to generate high uracil content DNA in *Drosophila* larval tissues. In addition, uracil-DNA was found to be present in all stages of development except in embryo. It indicates that uracil-DNA is not restricted to larval stages even though it is most pronounced in 3<sup>rd</sup> larvae.



Uracil level in *Drosophila melanogaster* samples is comparable to the uracil level previously reported in ung – /dut – *E.coli* [95]: 3000-8000 uracil/ 10<sup>6</sup> bases, or in other words, one out of every 125-300 bases is uracil. As DNA was isolated from the whole animal, these measurements could give an average estimation for uracil level present in the organism.

The highest value for uracil-DNA, 19000 uracil/ 10<sup>6</sup> bases (every 52<sup>nd</sup> base is uracil!) was found in salivary gland dissected from late 3<sup>rd</sup> stage larvae; which further supports accumulation of uracil in larval tissues (Figure 26C). Contrary to this, imaginal discs contain much less uracil. Results also indicate that uracil content varies according to the presence/absence of dUTPase in different tissues.

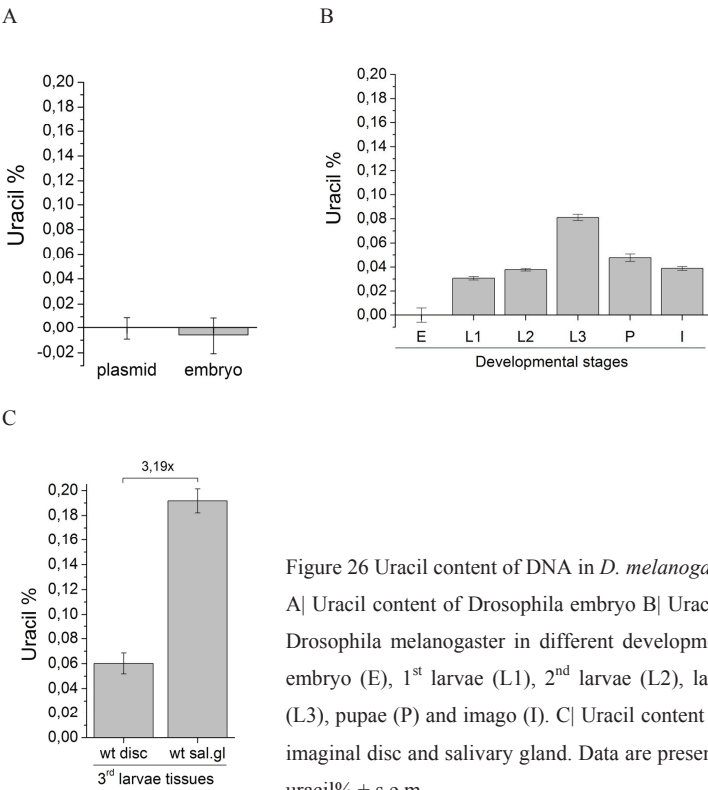


Figure 26 Uracil content of DNA in *D. melanogaster*  
A| Uracil content of *Drosophila* embryo B| Uracil content of *Drosophila melanogaster* in different developmental stages: embryo (E), 1<sup>st</sup> larvae (L1), 2<sup>nd</sup> larvae (L2), late 3<sup>rd</sup> larvae (L3), pupae (P) and imago (I). C| Uracil content of wild type imaginal disc and salivary gland. Data are presented as mean uracil% ± s.e.m.

This phenomenon was perturbed upon silencing of dUTPase in larvae which resulted in similar uracil content, as silencing increased the level of uracil in imaginal discs (Figure 27). Here I found clear correlation between the presence of dUTPase and uracil content of DNA. Even though dUTPase is present in wild type imaginal discs, according to these measurements, imaginal discs are not completely uracil-free, probably as a consequence of lack of UNG.

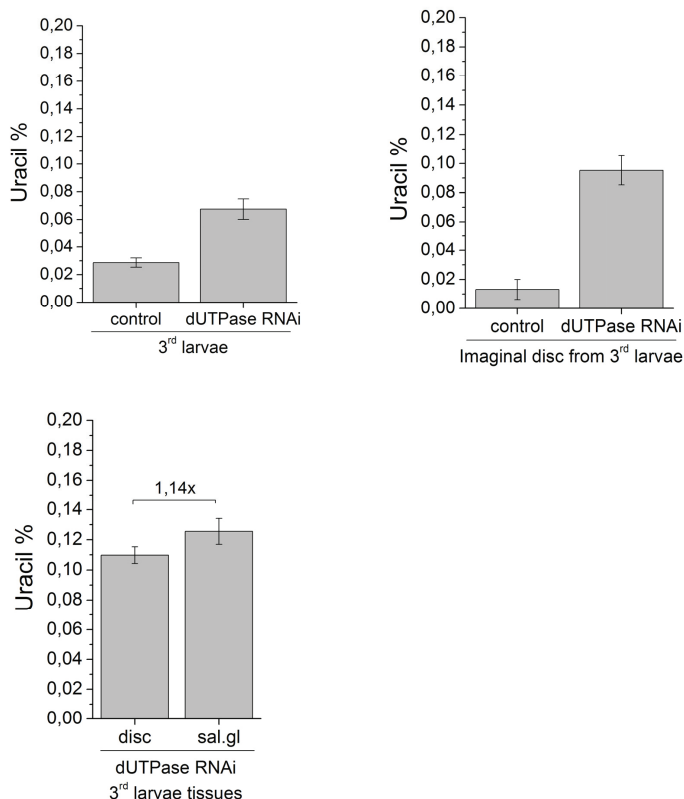


Figure 27 Silencing of dUTPase induced uracil accumulation also in the imaginal disc of *Drosophila melanogaste* larvae. Data are presented as mean uracil%  $\pm$  s.e.m.

Cell culture experiments further supports that 5'-fluorouracil (5'FU) and 5'-fluorodeoxyuridine (FUDR) treatment truly perturbed and increased the level of uracil which was well-tolerated by *Drosophila* Schneider 2 cells. Uracil incorporation resulted from 20-50 $\mu$ M 5'FU and 50-100 $\mu$ M FUDR was detected (Figure 28). In this concentration range 5'FU and FUDR are highly toxic to human cells because they leads to such a high level of uracil incorporation into DNA that induces DNA fragmentation thus thymine-less cell death. Such response to uracil-DNA was not provoked in *Drosophila* cells.

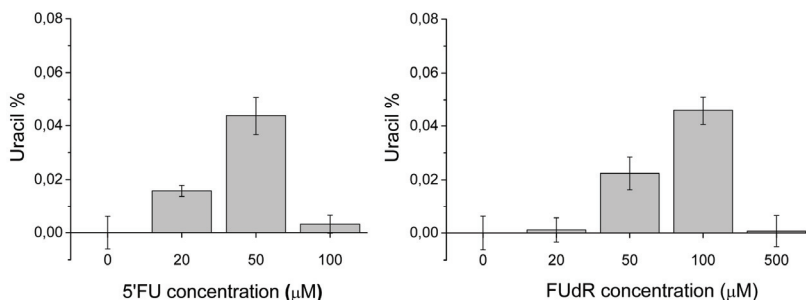


Figure 28 Thymidylate synthase inhibitors (5'FU and FUDR) caused uracil accumulation in the DNA of *Drosophila* S2 cells. Data are presented as mean uracil%  $\pm$  s.e.m.

## Discussion

### Subcellular localisation of *Drosophila* dUTPases

In this study, NLS of *Drosophila* dUTPase was identified *in silico* and confirmed *in vivo*. Recently, properties of *Drosophila* dUTPase NLS were further described in details. According to Merényi et al. [112] neutral and proline residues also contribute to the nuclear targeting potential of NLS. They showed that only the full length PAAKKMKID sequence able to direct clear nuclear accumulation of NLS-GFP construct, which is in agreement with the localisation pattern of the 23kDa dUTPase isoform. Truncated NLS (KKMKID), although it contained all basic residues, caused presence of NLS-GFP in the nucleus and in the cytoplasm as well. KMKID sequence was also able to target nucleus, even though lack of one of K residues. A subsequent lost of basic lysine (K) residue, as found in 21kDa isoform (MKID), leads to cytoplasmic localisation.

The dUTPase NLS segment is well preserved in other eukaryotic dUTPase sequences (Table 11) indicating that in most cases, the enzyme can be transported to its physiologically cognate cellular compartment, i.e. the nucleus.

Our present results confirm that in *Drosophila melanogaster* mitochondria does not contain dUTPase at detectable level. As mitochondria in fruitfly lacks UNG and also other uracil-DNA glycosylases, misincorporation of dUTP does not represent severe threat for the integrity of mitochondrial DNA as dUTP misincorporation-born uracil does not bear mutagenic potential. Theoretically regulation of dUTP/dTTP ratio could be achieved by the short (21kDa) dUTPase in the cytoplasm, and that ratio should be applied for the mitochondrial environment too.

Interestingly, *Drosophila* dUTPase may be excluded from the nucleolus (Figure 18), from the nuclear place of intensive ribosomal RNA synthesis, even though such restriction has not been observed in other localization studies. Nucleolus is freely available for most proteins present in the nucleus.

Localisation shifts of two dUTPase isoforms in *Drosophila* embryos of syncytial blastoderm stage show synchronised character (Figure 19). These shifts are closely timed to the nuclear cleavage phases (mitosis).

Shifts in localisation occur as nuclei enter mitosis and the nuclear pore complexes disassemble. The intact nuclear envelope also partially disintegrates, but remnants of it still provide some separation between nuclear and cytoplasmic compartments. This specific pattern has been termed as “semi-closed” (the term “semi-open” is also used) mitosis [113] and the retained nuclear membrane is called spindle envelope.

While nuclear pore complexes disassemble, the nuclear envelope becomes permeable. Permeability of the nuclear membrane give way to free passage of macromolecules into and out from the nuclear space. Size- and NLS-selective, directional importin-dependent active nuclear transport process through the pores can not operate from prophase [114].

As the border between nucleus and cytoplasm becomes permeable and non-restrictive, the 23kDa isoform diffuses out from the nucleus while the 21kDa isoform enters to the nuclear space and accumulates there. The observed accumulation of 21kDa isoform around the chromosomes in dividing nuclei (vs. diffusion of 23kDa isoform) could suggest that the 21kDa isoform might has an isoform specific interacting partner in the nucleus which is able to capture it and this putative interaction might be responsible for the unexpected localisation shift of 21kDa isoform. Identification of such an interacting partner and the selective manner of this hypothesised interaction with the 21kDa isoform would require further investigation.

The present results clearly indicate that the role of dUTPase isoforms in *Drosophila* is at least partially different from those described in humans with respect to the NLS-lacking isoform. In humans, this isoform is a “bona fide” mitochondrial protein [97], however, in *Drosophila*, the second isoform may also be present in the nuclear space, at least in the syncytial stage of embryonic development. During this stage, mitotic cycles follow each other in a very rapid manner. Our present results suggest that the presence of dUTPase in the nuclear space might be of increased importance during the whole cell cycle to provide accurate regulation of dUTP/dTTP pools for both repair and replicative DNA synthesis.

## Uracil-DNA in *Drosophila*: interpretation and developmental involvement

### Cellular response to uracil-DNA in *Drosophila melanogaster*

My results argue that a plasmid with high uracil-content (U-plasmid) can drive the expression of fluorescent protein in *Drosophila*, but not in human cells (Figure 20).

Uracil content of the reporter plasmid was similar in order of magnitude to the uracil content of genomic DNA of the adequate *E.coli* strain. XL1Blue *E.coli* produce DNA with uracil level under detection limit (1 uracil/ $10^6$  base), whereas ung-, dut- CJ236 *E.coli* strains synthesised DNA with 3000-8000 uracil/ $10^6$  base [95].

Such uracil content was sufficient for inducing cellular response to uracil-DNA and for distinguishing between different uracil-DNA-processing properties of *Drosophila* and human cells.

High uracil content of plasmid from CJ236 *E.coli* strain emerged mainly due to lack of dUTPase thus misincorporation of dUTP into the DNA. Such uracils in the A:U pairs are subjected to repair only by UNG. Even though expression level of all uracil-DNA glycosylases was not assessed for this experiment, results indicate that HeLa cells encoding ung gene are able to degrade U-plasmid, while lack of ung in *Drosophila* allows U-plasmid driven expression. It suggests that *Drosophila* cells (both, Schneider 2 cell culture and embryo) tolerate and interpret uracil-DNA and the other uracil-DNA glycosylases (SMUG, Thd1, MBD-R2) that are present in *Drosophila* could not substitute UNG in this assay.

Anticancer drugs, 5-fluorouracil (5'FU) and 5'-fluorodeoxyuridine (FdUR) affect the dUTP/dTTP ratio in the nucleotide pool and increases incorporation of dUMP into DNA. High level of uracil is subjected to DNA repair mainly by UNG which process leads to thymine less cell death. This is one of the mechanisms of their cytotoxicity and its importance is further underlined by the fact, that ung gene disruption in *S. cerevisiae* has protective effects against the lethality of 5-fluorouracil [115]. Similarly, lack of UNG in *Drosophila* has resulted in 5'FU and FdUR resistance/tolerance.

However, current results should be treated with caution. In human cells 5'FU can mediate its cytotoxic effect via two other routes as well. If converted to fluorodeoxyuridine triphosphate (FdUTP), it could get incorporated into the DNA, and subjected to similar DNA repair mechanism as uracil. If converted to 5-fluoro-UTP (FUTP), it could incorporate into RNA

molecules, particularly rRNA, which leads to inhibition of rRNA processing [116]. As a result of all above mentioned processes, 5'FU can induce cell cycle arrest and/or apoptosis. Impact of these mechanisms on overall cell death is a subject of current debate; however it possibly varies from cell line to cell line, and depends on the expression level of enzymes involved in nucleotide metabolism and uracil-repair [89, 117].

According to the literature, 5'FU or uracil from DNA (5'FU/U:A, 5'FU/U:G) is mainly removed by UNGs, but importance of the thymine DNA glycosylase (TDG) in mouse and human cells in 5'FU response was also reported. It was shown that inactivation of TDG is sufficient to confer resistance to 5'FU, whereas overexpression of TDG sensitizes cells to the drug [118].

Direct measurement of uracil content argues that 5'FU or FdUR treatment has led to dUTP/dTTP imbalance which has caused the misincorporation of dUMP during DNA replication. It gave rise to a dose-dependent increase in the steady-state level of uracil in DNA (Figure 28).

These results suggest that the genetic information stored in uracil-substituted DNA, plasmid or genomic DNA, serves as a cognate code for transcription in *Drosophila* cells. Such ability of the fruitfly cells is most probably due to lack of UNG so that uracil-DNA does not get rapidly degraded.

### **Significance of dUTPase in *Drosophila***

Although dUTPase is dispensable in larval tissues, the enzyme is essential in tissues preserved during metamorphosis for normal development. High level of uracil in DNA (synthesized in lack of dUTPase) might be tolerated only in stage and tissue-specific manner. Data argue that tolerance towards uracil-DNA might be restricted to larval stages and larval tissues.

Larval tissues containing high level of uracil-DNA are degraded during metamorphosis and imaginal discs with perturbed uracil-DNA content also showed developmental arrest. However, consequences and targeting mechanisms of uracil-DNA might be different in wild type larval and in dUTPase silenced imaginal tissues. Identification of such factors requires further investigation which would be highly important and interesting.

### **Uracil content of *Drosophila* biological sample**

The technique here applied gives numbers of uracil present in the PCR primer enclosed sequence and estimates the overall uracil-content of the genome.

Probably, level of uracil varies within different regions of chromosomes. Heterochromatin-euchromatin regions presumably differ in uracil content as a consequence of different condensation state, transcriptional activity, copy number or exposure to DNA damaging agents. Heterochromatin regions do not undergo endoreplication, remain condensed and most part of them is transcriptionally inactive [119]. Therefore lower rate of uracil accumulation could be presumed for these regions.

### **Protein factors putatively involved in response to uracil-DNA in *Drosophila melanogaster***

Uracil content measurements provided direct evidence that upon high dUTP level, during replication and DNA repair linked DNA synthesis high uracil content DNA is synthesised and it is maintained in *Drosophila* (Figure 26). These uracils are present in A:U pairs, therefore they are not mutagenic. They probably also serve as perfect code for RNA synthesis [120, 121], because RNA polymerase may also tolerate uracil in DNA.

In mammals, the major factor that cleave uracil from A:U context is UNG, thus in *Drosophila* tolerance of uracil-DNA is quite probably due to the lack of the *ung* gene. Above the absence of UNG from the whole organism, in larvae absence or low level presence of other uracil-DNA glycosylases could be presumed based on their expression level, which implies tolerance of G:U mispairs.

Enzymes involved in uracil-DNA repair pathways, such as *Drosophila* uracil-DNA glycosylases (SMUG, Thd1 and MBD-R2), AP endonuclease (Rrp1) and AP lyase (RpS3) [122] were scored whether their expression is turned off during larval stages [123].(Figure 28)

RNA level of most uracil-DNA repair enzymes is relatively high in embryo and low in larvae according to microarray based data published on Flybase [123, 124] (Table 14). However enzymes responsible for other DNA damage repair processes are expressed in different pattern, some even show higher level in larvae than in embryo (Table 15). Therefore we could assume that low level of expression is not ubiquitous among proteins involved in DNA repair pathways but characteristic to uracil-DNA repair. Thd1, the only uracil repair enzyme showing balanced expression throughout development, repairs U:G mismatches, thus it does not act on uracil resulted from dUTP incorporation. Taken together, three different factors



were described which are responsible for stage-specifically elevated level of uracil in larvae:  
1) lack of ung gene, 2) absence of dUTPase protein and 3) decreased level of RNA of enzymes working on uracil removal.

human	Drosophila	expression in Drosophila larvae
UNG	-	-
dUTPase	dUTPase	↓ in larvae
SMUG	SMUG	↓ in larvae
TDG	Thd1	not specific
MBD4	MBD-R2	↓ in larvae
APEX1, APEX2	Rrp1	↓ in larvae
RpS3	RpS3	↓ in larvae

Table 14 Expression levels of enzymes involved in uracil-DNA repair are lower in larvae than in embryo.

Base Excision Repair	SMUG, MBD-R2	Uracil-DNA glycosylases	↓
	Thd1 (TDG)	Uracil DNA glycosylases	≈
	CG9272	Oxidized pyrimidine base lesion	↓
	Ogg1	Oxidized purine base lesion	≈
	Rrp1, RpS3	AP endonucleases	↓
	XRCC1	damaged DNA binding	↓
	PARP	DNA binding	↓
	FEN1	endonuclease activity	≈
Nucleotide Excision R.	mus201	ssDNA endodeoxyribonuclease	↓
	CG10670	XPG-like endonuclease	≈
	XPA	damaged DNA binding	↓
	XPB	DNA helicase	↑
Mismatch Repair	Mlh1 (MutL)	mismatched DNA binding	≈
	Pms2 (MutL)	mismatched DNA binding	↑
	Msh6 (MutS)	mismatched DNA binding	↑
	mus209 (PCNA)	recruitment	≈
DNA break	CG5247 (Ku70/Ku80)	DNA helicase	↑
	Rad50	nuclease	↓

Table 15 Low level of expression is not ubiquitous among proteins involved in DNA repair.

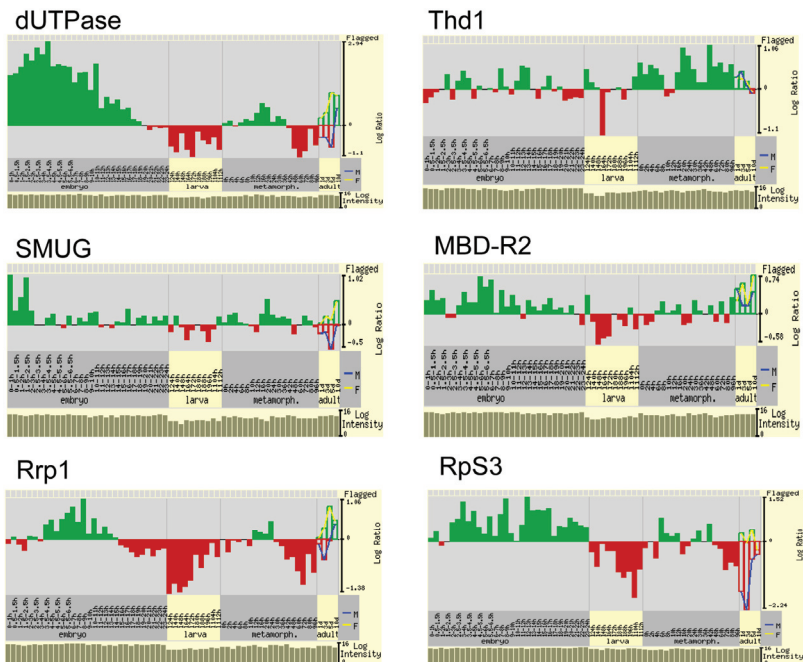


Figure 29 Expression patterns of uracil-DNA repair genes in *Drosophila melanogaster*. [123]

The extraordinary situation of tolerance and interpretation of uracil-DNA may not be exclusively present in *Drosophila* as absence of *ung* is ubiquitous among Holometabola (Table 16). As uracil-DNA naturally occurs in larval tissues that are sentenced to death, we consider that uracil-DNA might be linked to metamorphosis and tissue degradation in a general fashion in Holometabola insects. Further investigations should be taken to describe the mechanism, its impact and its putative role.

		dUTPase	UNG
Insecta	Holometabola		
	Diptera		
	<i>Drosophila melanogaster</i> (fruitfly)	+	-
	<i>Culex pipiens</i> (mosquito)	+	-
	<i>Aedes aegypti</i> (mosquito, yellow fever)	+	-
	<i>Anopheles gambiae</i> (mosquito, malaria)	+	-
	<i>Bombyx mori</i> (silkworm)	+	-
	<i>Tribolium castaneum</i> (red flour beetle)	+	-
	<i>Apis mellifera</i> (honey bee)	+	-
	<i>Nasonia vitripennis</i> (parasitoid wasp)	+	-
	<i>Acyrtosiphon pisum</i> (pea aphid)	+	-
	<i>Pediculus humanus corporis</i> (body louse)	+	+

Table 16 The gene of the main uracil-DNA glycosylase, *ung* is not encoded in the genome of Holometabola species.

The main message of this work is that uracil-substituted DNA can be tolerated and interpreted in *Drosophila* that is further paralleled by stage and tissue-specific presence of dUTPase and uracil-DNA repair pathways.

## Further Speculations and Open Questions

Lack of UNG in *Drosophila* and Holometabola group of insects is exceptional among living organisms, but presence of three uracil-DNA glycosylases with different enzymatic properties might at least partially substitute UNG function to keep mutation rate at low level.

dUTPase and Thd1 are enriched in embryonic gonads [125], therefore Thd1, G:U mispair-specific uracil-DNA glycosylase, might be able to preserve integrity of DNA and dUTPase supposedly assure that DNA is synthesised in a relatively dUTP misincorporation-free fashion.

The major threat of uracil lays in its mutagenic potential, if it occurs in mismatch with guanine. A:U pairs were not reported to be harmful and uracil-DNA glycosylases in *Drosophila* might not be available or not be able to process them. In such cases, presence of A:U pairs would only depend on dUTPase activity.

Surprisingly, dUTPase is not expressed in larval tissues and many other tissues of imago, only detected in embryo, imaginal tissues and ovaries [100]. According to microarray data, its mRNA level is also elevated in CNS (central nervous system) of larvae, but these tissues resemble to imaginal tissues in respect of keeping diploid chromosomal state and do not go through rapid degradation during metamorphosis. Microarray data also indicates that expression of SMUG, MBD-R2 and Rrp1 show similar tissue- and stage- specific pattern as dUTPase, however Thd1 might be an exception.

SMUG might be able to cut either deamination-born or misincorporation-born uracil in single-stranded DNA environment (Figure 9). The observed severe developmental defects in dUTPase silenced animals could be also explained theoretically by SMUG induced DNA damage response.

Stage- and tissue- specific pattern of dUTPase expression and presence of uracil-DNA might argue for their developmental importance. According to uracil-DNA level measured in ung- *E.coli*, lack of UNG would not be sufficient alone for such high level of uracil accumulation.

Table 17 summarises some of the possible causes and consequences of high level of uracil in DNA under physiological conditions in *Drosophila* larvae. Furthermore, it aims to inspire further investigations and considerations on this exciting phenomenon on the narrow margin of DNA metabolism and *Drosophila* development.

Causes	Uracil-DNA	Possible consequences
Lack of UNG		Uracil-DNA cleavage, degradation
Lack of dUTPase		DNA damage response, apoptosis, cell cycle arrest
SMUG, TDG, MBD-R2 may not act efficiently on A:U basepairs Errors introduced during DNA synthesis and transcription coupled DNA repair		Transcription misregulation: No binding of transcription factors

Table 17

## Epilogue

In the micro-cosmos of *Drosophila melanogaster*, two aspects of uracil-DNA metabolism were discovered. First, novelty of dUTPase localisation in early embryo caused amazement. In this state of development dUTPase is present with high protein level preventing incorporation of dUTP into DNA. Second, uracil measurement in larvae provided supportive evidence for the astonishing hypothesis that uracil-DNA is accumulated in larval tissues as a consequence of lack of dUTPase and lack of UNG.

Special characteristics of *Drosophila* uracil-repair probably harbour more to explore.

## Summary

Uracil frequently appears in DNA either from cytosine deamination or thymine-replacing incorporation, even though it is not a native component of DNA. Catalytic actions of two key factors are responsible for maintaining uracil-free DNA: dUTPase prevents uracil (dUTP) incorporation into DNA, and uracil-DNA glycosylases excise uracil from DNA. Prevention and removal of uracil highly contribute to preserving the genetic information encoded in DNA.

In this study, appearances of uracil have been investigated in *Drosophila melanogaster*. Most importantly, I confirmed that larval tissues accumulate uracil in DNA, as both prevention and removal of uracil is impaired because the major uracil-DNA glycosylases (UNG) is not encoded in the genome of *Drosophila melanogaster* and dUTPase is down-regulated in larval tissues. By applying multiple approaches, I showed that *Drosophila* cells are capable of tolerating high level of uracil in DNA. Lack of UNG in all examined Holometabola species indicates potential wide presence of uracil-DNA in the insect world. These data open exciting possibilities for the putative role of uracil-substituted DNA in insect development and metamorphosis.

As ung is not encoded in *Drosophila melanogaster*, significance of dUTPase in uracil metabolism might go beyond expectations. Therefore much attention was paid to dUTPase in the fruitfly.

In such experiments where dUTPase RNAi was induced, data argues that dUTPase is not dispensable in *Drosophila*, and lack of dUTPase in imaginal tissues leads to lethality.

Studies on uracil content aimed to confirm the impact of dUTPase in such emerges. To achieve it, a recently developed technique was used to quantify uracil content of DNA. Results showed that lack of dUTPase indeed increases the level of uracil in DNA.

Two dUTPase isoforms (23 kDa and 21 kDa) are present in the fruitfly. I confirmed that the PAAKKMKID sequence of 23kDa isoform N terminal region is an NLS, and 23kDa isoform is present mainly in the nucleus in *Drosophila* Schneider 2 cells. The 21kDa isoform lacking NLS stayed in the cytoplasm, indicating that no mitochondrial isoform of dUTPase exists in *Drosophila*. Surprisingly, upon microinjection of 21kDa dUTPase isoform into actively

dividing *Drosophila* embryos, it accumulated in the nuclear space at certain stages of mitosis. The observed dynamic localisation character showed strict timing to the nuclear cleavage phases and indicated that both isoforms can be present within the nuclear microenvironment, although at different stages of cell cycle.



## Összefoglalás

A DNS-t adenin, timin, guanin és citozin bázisok építik fel. Uracil csak elvétve fordul elő benne: ha a DNS polimeráz hibát vét és dUTP-t használ fel dTTP helyett a DNS szintéziséhez, vagy, ha egy-egy citozin bázis elveszíti aminos csoportját (deaminálódik). Kétféle mechanizmus óvja a DNS-t az uracil jelenlététől, egyrészt a dUTP-t bontó dUTPáz megelőzi a beépülését, másrészt az uracil-DNS glikozilázok eltávolítják a „hibás” bázist. Mindkét folyamat nagymértékben hozzájárul a DNS-en kódolt genetikai információ megőrzéséhez.

Doktori értekezésemben az uracil-DNS megjelenésének lehetőségeit vizsgáltam ecetmuslicában. Elsőként kiemelném, hogy sikerült bizonyítanunk, hogy az ecetmuslica lárvális szöveteiben jelentősen megnő a DNS uracil tartalma. Ennek oka a dUTPáz és a legnagyobb katalitikus hatékonysággal bíró uracil-DNS glikoziláz (UNG) aktivitásával magyarázható. A dUTPáz nem fejeződik ki a muslica lárvális szöveteiben, valamint az UNG-t kódoló gén hiányzik az ecetmuslica genomjából. Többféle megközelítést alkalmazva bebizonyítottam, hogy a muslica sejtek képesek tolerálni az uracil magas szintjét is a DNS-ben. Mivel az UNG-ot kódoló szekvencia nemcsak a muslicából, de az összes megvizsgált teljes átalakulással fejlődő rovar (Holometabola) genomjából is hiányzik, az uracil-DNS általánosan előfordulhat a Holometabola lárvák DNS-ében.

UNG hiányában a dUTPáznak kiemelten fontos szerep juthat a DNS uracil tartalmának szabályozásában, ezért további kutatásaim a dUTPáz enzimmel foglalkoztak.

dUTPáz csendesítés hatására a dUTPáz fehérje eltűnt az imaginalis diszkuszokból, ami letális fenotípust okozott, valamint dUTPáz hiányában megnövekedett a DNS uracil tartalma.

Ecetmuslicában, csakúgy, mint emberben két dUTPáz izoforma található meg (21kDa és 23kDa). Bebizonyítottam, hogy a 23kDa-os izoforma N-terminálisán található PAAKKMKID szekvencia a nukleáris lokalizációért felelős nukleáris lokalizációs szignál (NLS). Ezzel ellentétben, a 21kDa-os izoforma nem hordozza ezt az NLS-t és a sejt kultúrában végzett kísérletek alapján a citoplazmában lokalizálódik. Különösen érdekes, hogy ecetmuslicában nincsen mitokondriális izoformája a dUTPáznak. Meglepő módon, a 21kDa-os izoforma a sejt magosztódások során felhalmozódik a sejt magnak megfelelő térrészben *Drosophila melanogaster* korai embrióban. A dUTPáz lokalizációjának változása szigorúan a sejt ciklus

bizonyos fázisaihoz kötött és biztosítja, hogy mindkét izoforma előfordulhat a sejtnek megfelelő térben, bár a sejtciklus eltérő fázisaiban.

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## Publication list

### Journal articles

V. Muha, I. Zagyva, Z. Venkei, J. Szabad, B.G. Vertessy, *Nuclear localization signal-dependent and -independent movements of Drosophila melanogaster dUTPase isoforms during nuclear cleavage*, Biochem Biophys Res Commun 381 (2009) 271-275.

Bekesi, M. Pukancsik, V. Muha, I. Zagyva, I. Leveles, E. Hunyadi-Gulyas, E. Klement, K.F. Medzihradzsky, Z. Kele, A. Erdei, F. Felfoldi, E. Konya, B.G. Vertessy, *A novel fruitfly protein under developmental control degrades uracil-DNA*, Biochem Biophys Res Commun 355 (2007) 643-648.

### Manuscript submitted for publication

V. Muha, A. Horváth, M. Erdélyi, M. Pukáncsik, A. Békési, B. Hodoscsek, G. Merényi, F. Jankovics, B.G. Vértessy, *Uracil-DNA in Drosophila: interpretation and developmental involvement*

## Selected Presentations

### Poster

Muha V, I. Zagyva, Zs. Venkei, J. Szabad and B. G. Vértessy "Different nuclear localisation mechanisms for the two *Drosophila* dUTPase isoforms". 31<sup>st</sup> FEBS Congress, Istambul, Turkey, June 24-29, 2006

Muha V, A. Békési, I. Zagyva, B. G. Vértessy "From uracil-DNA to cell death within the context of fruitfly metamorphosis" Alexander von Humboldt Workshop on Structure-Based Approaches Towards Disease Control, Mátraháza H, 2007

Muha V. and B.G. Vértessy "siRNA silencing of dUTPase and uracil-DNA nuclease, key factors in uracil-DNA metabolism, perturbs development of *Drosophila melanogaster*" Annual Meeting of the Hungarian Biochemical Society, Szeged H, 2008

### Oral

Muha V, B Hodoscsek, and B.G. Vértessy "The new world of uracil-DNA" Straub Meeting of the Biological Research Center, Hung. Acad. Sci., Szeged H, 2008